SMALL DIAMETER VASCULAR PROSTHESES

Experimental Studies on Prevention of Thrombosis

Carlos O. Esquivel

Malmö 1982

Lund

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Legend to the cover:

Scanning electron micrograph. Distal anastomosis of a nonheparinized polyurethane graft showing early thrombosis. Distorted erythrocytes, platelets and fibrin-like material are observed. X1000

UNIV. LEIDEN BIBL.

This dissertation is based on the following articles:

I. Carson S, Demling R, Esquivel C, Talken L, Tillman P:
Testing and treatment of arterial graft thrombosis.
Am J Surg 142:137-143, 1981.

II. Esquivel CO, Björck C-G, Bergqvist D, Carson SN, Rothman U, Bergentz S-E:
Decreased acute thrombogenicity of human umbilical veins after heparin and alcohol treatment.
Eur Surg Res. In press.

III. Esquivel CO, Björck C-G, Bergentz S-E, Bergqvist D, Larsson R, Carson SN, Dougan P, Nilsson B:
Reduced thrombogenic characteristics of expanded polytetrafluoroethylene and polyurethane arterial grafts following heparin bonding.
Submitted for publication.

IV. Esquivel CO, Björck C-G, Bergqvist D, Bergentz S-E:
Heparinized human umbilical vein grafts: Delayed heparin desorption after alcohol treatment.
Submitted for publication.

V. Esquivel CO, Bergqvist D, Björck C-G, Nilsson B:
Comparison between commercial heparin, low molecular weight heparin and pentosan polysulfate on hemostatis and platelets in vivo.
Thromb Res. In press.

VI. Carson SN, Esquivel CO, French SW:
Experimental carotid stenosis due to fibrous intimal hyperplasia.
Surg Gynecol Obstet 153:883-888, 1981.

VII. Esquivel CO, Björck C-G, Saldeen P, Ljungnér H, Bergqvist D:
Prostacyclin (PGI_2) production and fibrinolytic activity after experimental vascular grafting.
Submitted for publication.

VIII. Esquivel CO, Bergqvist D, Björck C-G, Carson SN, Nilsson B:
Assessment of antithrombotic properties of sodium ibuprofen.
Thromb Haemost 48:87-90, 1982.

The above articles will be referred to in the text according to their Roman numerals.

Abbreviations

HUV	= Glutaraldehyde-tanned, human umbilical vein grafts
PTFE	= Expanded polytetrafluoroethylene grafts
PU	= Polyurethane grafts
HAUV	= Heparin and alcohol-treated human umbilical vein grafts
HsAUV	= Heparin without alcohol-treated human umbilical vein grafts
NHUV	= Nonheparinized human umbilical vein grafts
Hep-glut PTFE	= Glutaraldehyde-stabilized ionic bonding of heparin onto PTFE grafts
Hep-glut PU	= Glutaraldehyde-stabilized ionic bonding of heparin onto PU grafts
Cov-hep PTFE	= Covalent bonding of heparin onto PTFE grafts
FIH	= Anastomotic fibrous intimal hyperplasia
PGI_2	= Prostacyclin
PA	= Plasminogen activator

Contents

Page

6 Abstract
7 INTRODUCTION
8 NEED FOR VASCULAR PROSTHESES
8 Historical remarks
9 BLOOD AT INTERFACE
9 Interaction to natural surface
10 Interaction to artificial surface
12 CURRENT VASCULAR PROSTHESES
12 Grafts of biologic origin
13 Synthetic grafts
13 Graft performance in clinical practice
15 Causes of graft failure
16 Methods to improve outcome
19 PURPOSE
19 METHODS AND MATERIALS
19 Graft performance
23 Methods to reduce thrombogenicity
25 Other factors which may influence graft performance
27 Experimental design
28 Statistical analysis
29 RESULTS
29 Graft performance
30 Other factors which may influence outcome
36 GENERAL DISCUSSION
36 Heparin bonding to biologic (HUV) grafts
38 Heparin bonding to synthetic grafts (PU and PTFE)
39 Anastomotic fibrous intimal hyperplasia
40 Prostacyclin (PGI_2) and plasminogen activator (PA)
42 Adjuvant antithrombotic therapy
46 CONCLUSIONS
48 REFERENCES
63 ACKNOWLEDGEMENTS
ARTICLES I-VIII

Abstract

Thrombosis is a common cause of failure of small diameter vascular prostheses (<6mm). The purpose of this work was to study the effect of heparin bonding and the administration of an antiplatelet drug (ibuprofen) on graft performance. The following factors which may influence the outcome were also investigated: heparin desorption, effect of heparin on platelet reactivity in vivo, development of anastomotic fibrous intimal hyperplasia (FIH) and the production of PGI_2 and plasminogen activator (PA) from the anastomoses and midgraft regions. Methods: Grafts were placed in the carotid arteries of sheep or dogs at different flow rates and removed 4 hours, 10 or 90 days later. The thrombogenicity of the graft material was assessed by determining the thrombus-free surface, labelled-platelet deposition in vivo, thrombus weight and patency. Results: The combination of heparin and alcohol bonding significantly reduced the thrombogenicity of modified human umbilical vein (HUV) compared to heparinization without alcohol or no heparinization. The alcohol treatment significantly delayed the heparin desorption over time although it did not improve graft patency in the 10 day experiments. Stabilized ionic bonding of heparin onto polyurethane (PU) grafts significantly diminished their thrombogenicity compared to untreated ones but had little effect on polytetrafluoroethylene (PTFE). The thrombogenic characteristics of the latter were, however, reduced by covalent bonding of heparin. Bonded heparin decreased labelled-platelet deposition but increased the platelet reactivity in vivo after intravenous administration in rabbits. Anastomotic FIH developed after insertion of interposition PTFE grafts or endarterectomies with PTFE angioplasties. Heparin bonding to HUV appeared to limit the progression of the lesion. A variable PGI synthesis was noticed from the anastomotic sites and seemed to depend on the graft patency and grafting site. HUV neointima acquired the ability to produce PGI_2 and PA. Ibuprofen decreased platelet function in vivo and the weight of thrombi ex vivo. It also diminished the thrombogenicity of PTFE grafts.

Key words: Thrombosis; Thrombogenicity; Heparin bonding; Heparin, Platelets; Ibuprofen; Vascular grafts; Anastomotic intimal hyperplasia; PGI_2; Plasminogen activator

INTRODUCTION

The number of vascular reconstructive procedures has increased considerably in the last two decades. Callow recently calculated that approximately 550,000 graft implantations are performed in the United States annually[38]. In Sweden, with a population of 8 million, approximately 3,900 reconstructive procedures were performed in 1980 and in Malmö with 250,000 inhabitants where there is a good capacity for and interest in vascular surgery, the number of vascular reconstructions has increased from 103 to approximately 450 per year from 1971 to 1981[19,21]. These facts in turn, have lead to an increasing demand for arterial substitutes to replace diseased vessels.

Poor results are usually encountered with small diameter prostheses (<6 mm) particularly when they are used in conditions of high resistance and low flow, for instance, in distal vessels of the lower extremity[67,82,110,219,243,250]. An adequate blood flow is fundamental for a good outcome. Poor inflow or outflow leads to inevitable graft loss. Failure of small diameter vascular prostheses can be classified as early and late. Factors incriminated in early failures are: technical errors[72,151] and the thrombogenicity of the graft material[82,135,210]; in late failures: development of anastomotic fibrous intimal hyperplasia[81,124,194], false or true aneurysms[57,97,169,170], infection[43,100] and progression of arteriosclerotic disease[38,223,246]. The best arterial substitute is probably the autogenous artery but because of its limited availability, it is seldom used. The best alternative is the autogenous vein (principally the saphenous vein)[67,74,76,82,243,250]; nonetheless, it cannot always be used due to gross abnormalities or because it might have been removed in a previous operation. Moreover, autogenous veins might develop late complications such as aneurysmal dilatations, fibrosis and intimal hyperplasia[151,225,226]. Therefore, there is a tremendous need for an arterial substitute which would perform as well as or better than the autogenous veins.

The general purpose of this investigation was to study methods to reduce the thrombogenicity of commercially available grafts (HUV and PTFE) and an experimental graft (polyurethane material) by means of heparin bonding or by the administration of an antiplatelet drug (ibuprofen).

NEED FOR VASCULAR PROSTHESES

Historical remarks

In the beginning of this century, Carrel and Guthrie approached the problems of technique in vascular surgery. They worked with vascular grafts, particularly homologous vein grafts in experimental animals and emphasized the importance of the anastomosis as the initiator of thrombus formation[41]. It appears that the first successful arterial grafting using an autogenous vein was that reported by Goyanes in 1906[102]. A segment of the popliteal vein was used to restore continuity of the femoral artery after resection of an aneurysm. Bernheim in the United States used a segment of the saphenous vein to bridge the defect created by excision of a popliteal aneurysm[160]. Nevertheless, ligature remained the standard method of treatment for arterial injuries. Because of the high incidence of amputation during World War II - 62% if only wounds of the iliac, femoral and popliteal arteries were considered[69] - attempts were made to avoid suture anastomoses and instead vitallium tubes were used but with very disappointing results[27].

Meanwhile, with the development of arteriography[78], it became apparent that arteriosclerosis was, by and large, a segmental disease and surgical correction was therefore possible. Heparin had been discovered[120], and the first reports of its usefulness in the prevention of thrombosis in arterial surgery began to emerge [176,177]. An adequate arterial substitute became an obvious necessity.

Soon the replacement of arteries with allografts gained popularity[107]; however, discouraging results in the literature began to appear[227]. As should have been expected, degenerative changes in the allografts due to immunologic rejection ensued within two years leading to graft failure[242]. These poor results stimulated the search for a new arterial substitute.

Because of the unsatisfactory results using rigid glass and metal tubes obtained by Carrel, Guthrie and Touffier, attention was directed towards the use of new plastic materials[236]. In 1952, Voorhes successfully implanted plastic prostheses (Vinyon N) in dogs[237]. This was a porous material which would allow healing and incorporation of the graft in the host tissues with the forma-

tion of a neointima. Several materials were then tried: Nylon, Teflon, Orlon and Dacron and the first review was reported in 1957[60].

The results using plastic fabrics were not entirely satisfactory particularly with the small diameter prostheses and led surgeons' attention to biologic materials. Rosenberg et al.[198] introduced the bovine heterograft which was later modified to provide it with a negatively charged surface[214].

Eiken and Nordén developed a technique for the formation of fibrocollagenous grafts in situ by implanting plastic rods adjacent to the arteries of dogs[85]. A similar principle was used by Sparks who developed the methods for creation of fibrocollagenous grafts[218]. An early success was achieved with the use of this graft in humans[217]; however, late complications such as difficulties in maturation, initial thrombosis (17%), late thrombosis (44%), postoperative bleeding and aneurysm formation led to its abandonment in clinical practice[111].

BLOOD AT INTERFACE

Interaction to natural surface

Endothelium

Blood compatibility has been defined as the inability of a surface to attract or alter cellular elements or to induce blood coagulation[158]. The only blood compatible surface known thus far is the endothelium. The exact mechanism by which the endothelium prevents thrombus formation is not fully understood at the present time; nonetheless, some of its antithrombogenic characteristics have been reported: the presence of glucosaminoglycans[37,137] and antithrombin III[45], specific binding for heparin[98], and for thrombin[7], and production of plasminogen activators and prostacyclin (PGI_2)[125,171,232]. This subject is reviewed elsewhere[96].

Subendothelium and collagen

The reaction of platelets with subendothelium and collagen has also been reviewed in detail by others[13,128,168]. There appear to be some differences between the reaction of platelets with collagen compared with subendothelium[14]. For instance, calcium is

necessary for the platelet reaction with subendothelium but not with collagen. Moreover, several conflicting reports regarding the collagen platelet reaction have appeared in the literature[159]. It appears that collagen from different parts of the body reacts differently with platelets because of its different biochemical structure. The presence of glycosaminoglycans also seems to alter the collagen activation of the intrinsic blood coagulation[158].

Interaction to artificial surface

A series of events occurs as soon as the blood comes in contact with a foreign surface. These events involve adsorption of plasma proteins, adhesion and aggregation of platelets as well as adhesion of other blood constituents. As a result, thrombus formation or consumption of clotting factors and blood elements may ensue [91,92].

Adsorption of plasma proteins

Adsorption is the initial event which takes place within seconds after contact of blood with a foreign surface. Adsorption of proteins occurs when the foreign surface is exposed to whole blood, plasma or purified protein solutions and also depends on the specific properties of the artificial surface. Several comprehensive reviews of this phenomenon are available[32,139, 239].

Fibrinogen, Factors XII, XI, V and VII and thrombin are rapidly adsorbed[9,48,156,234,238]. Some of these proteins can trigger the intrinsic blood coagulation and induce platelet adhesion[32,251].

Other proteins are also adsorbed: albumin, α-, β- and γ-globulins, transferrin and ceruloplasmin[92]. It appears that adsorbed fibrinogen increases platelet reactivity[207,251] whereas albumin coated surfaces reduce platelet activity[154,208].

Hydrophobic polymers are reported to adsorb more proteins than hydrophilic ones[33]. Hydrophobic polymers are so called because water or aqueous fluids on their surfaces will form a drop having a nonzero contact angle. They do not absorb water (usually <0.1%) and have successfully been used in medical devices such as catheters and intraaortic ballons[31]. Most of the vascular prostheses in the market belong to this category of polymers.

Hydrophilic polymers are defined as polymers which absorb and retain water in their structure (e.g. >10-20%) and do not dissolve in water[118]. In a recent report, Uniyal and Brash demonstrated that fibrinogen and albumin were not adsorbed onto hydrophilic surfaces and except for polystyrene, albumin was adsorbed onto hydrophobic surfaces but not fibrinogen[234]. As previously mentioned, the adsorption of albumin may have an antithrombotic effect by impedement of platelet adhesion.

Adhesion of platelets

Platelets are the most studied blood elements in the interaction with foreign surfaces[50,206,207]. As mentioned before, platelets interreact with the adsorbed proteins in the surface, particularly fibrinogen and γ-globulins[89,207,251]; whereas an albumin-coated surface discourages platelet adhesion[154,208]. In vascular prostheses, platelets may induce thrombus formation leading to occlusion or may form aggregates or small thrombi followed by embolization[88,211]. Moreover, Harker has also shown a decreased platelet survival induced by Dacron grafts in humans and experimental animals[113].

The specific characteristics of the surface seem to influence the adhesiveness of platelets. Hydrophobic surfaces attract platelets more than hydrophilic surfaces; however, surfaces that support adhesion induce little release and surfaces that do not support adhesion seem to induce a considerable release reaction[31]. Conflicting reports exist in the literature in regards to the effect of heparinized surfaces on platelets[147,208]. It appears, though, that heparinized surfaces do not prevent platelet adhesion completely but they do reduce platelet adhesion compared to nonheparinized ones[143].

Sawyer and Pate introduced the term "surface charge". They suggested that the endothelium is blood compatible because of its negative charge and will repel the platelets since they also have a negative charge[213]. In experimental animals the use of electrical current delayed thrombosis[212]; nonetheless, negatively charged surfaces have been found to activate F-XII and induce blood coagulation[121,180]. Furthermore, platelets can adhere to negatively and positively charged surfaces[180,208]. Much is known about the adhesion of platelets to subendothelium and foreign surfaces and very thorough reviews have been reported elsewhere[158,178,205].

Adhesion of other blood elements

The interaction of other blood constituents to foreign surfaces has not been studied as thoroughly as with platelets. Leukocytes are known to possess a procoagulant activity[179]. This activity has been demonstrated in polymorphonuclear leukocytes, lymphocytes and macrophages[94,150,203]. Furthermore, leukocytes produce thromboxane A_2, a potent platelet aggregating substance[99].

Red blood cells (RBC's) have been considered passive elements in the formation of a thrombus and it is felt that they become trapped in the fibrin strands. With the advent of scanning electron microscopy (SEM), it has been shown that RBC's also adhere to a foreign surface despite a minimal amount of fibrin, indicating that the cells become "sticky". The exact mechanism of this phenomenon is unclear; although, it is thought that RBC's participate in the thrombus formation process by providing phospholipids and ADP. A good review of the interaction of leukocytes and RBC's to artificial and natural blood vessels was done by Stewart et al.[221].

CURRENT VASCULAR PROSTHESES

Grafts of biologic origin

Modified bovine grafts

Bovine grafts are obtained from the carotid arteries and treated with a proteolytic enzyme for 2½ hours at 37° C for digestion of the muscular and elastic layers rendering them non-antigenic[70]. Subsequently, the graft is placed in a glass rod, tanned with a 1.3% dialdehyde starch solution and then stored in 40% alcohol. Encouraging reports appeared in the literature in the early seventies[136]. In the femoral popliteal location the patency rate was reported as 68% for above knee and 17% for below knee reconstruction[199]. However, the use of bovine heterografts has declined because of the risk of aneurysm formation, decreased resistance to infection and high cost.

Human umbilical cord vein grafts

Dardik and Dardik introduced the modified human umbilical cord vein (HUV) graft as an arterial substitute in the midseventies[66]. In order to prevent aneurysmal formation and rejection, the HUV is

tanned with glutaraldehyde and wrapped in polyester mesh. Glutaraldehyde as a tanning agent was found to be superior to dialdehyde[65].

Synthetic grafts

Dacron

Vascular prostheses made of Dacron have been designed in different ways, e.g. woven, woven elastic, knitted, crimped or uncrimped. Suffice it to say that Dacron grafts have been successfully used in the aortoiliac (femoral) region[224], but disappointing results have frequently been encountered when used in the lower extremety[228].

Expanded polytetrafluoroethylene

Expanded PTFE is a porous hydrophobic polymer with a highly electronegative surface[39,161]. Because of the incidence of aneurysms, the graft has been reinforced with two PTFE structures[39]. It is somewhat rigid which might account for some of the complications such as avulsion of the anastomoses[4] or development of anastomotic intimal fibrous hyperplasia[42,81]. It is impervious which means that it does not require preclotting and the risk of blood loss or hematoma formation is therefore reduced[4].

Graft performance in clinical practice

Bovine, PTFE and HUV grafts have been used as arteriovenous fistulas[202,204] and some of these grafts have been used in other locations e.g. axillofemoral, femorofemoral, aortocoronary and aortopulmonary bypasses[4,56,108]; however, it is out of the scope of this manuscript to discuss graft performance in those locations. Currently, PTFE and HUV's are the vascular substitutes most commonly used in revascularization of the lower extremity when an autogenous vein graft is not available. Henceforth, I will only discuss these two types of grafts.

Good results have been reported using PTFE in peripheral arterial reconstructions[39,90,108,235]. A 72% cumulative patency rate including reoperated grafts was achieved by Veith, Gupta and Daly

but considering the 27 reoperated cases as failures, the patency rate dropped to 62% at 3 years. Moreover, their patients received aspirin (ASA) and dipyridamole which may have had a beneficial effect[235]. Likewise in Campbell's series some of the patients received antiplatelet therapy also[39]. Evans et al. reported a similar patency rate at 3 years (58%) which fell to 48% at 4 years [90]. It appears, though, that their patients were in a better risk population since in 47% the runoff was graded as good. In patients with good runoff, Hearn and Charlesworth obtained an acceptable patency rate, almost similar to the autogenous saphenous vein, but in patients with poor runoff 100% failure rate was obtained[115].

In a recent review, Dardik et al. reported a 60% cumulative patency rate for femoral popliteal bypass at 5 years, 38% for femorotibial bypass at 4 years and 28% for femoroperoneal bypass at 4 years using the HUV [64].

Because of variation in the patient population, the individual selection of patients and the surgeon's experience with a specific vascular prosthesis, a prospective randomized study comparing the different types of grafts by the same surgical team would be ideal.

Although including several surgical teams in different hospitals, a prospective study comparing the HUV and PTFE in femoropopliteal bypass procedures was recently performed in the Scandinavian countries. The study was based only on the first graft failure; therefore, reoperated cases were not considered. The cumulative patency rate for PTFE and HUV at 22 months was 40% and 75%, respectively and the difference was statistically significant ($p=0.014$)[84].

There are a few retrospective studies comparing the saphenous vein to the HUV and PTFE vascular grafts. In a clinical study by Cranley and Hafner, there were 3 to 4 times more early failures (within 1 month after surgery) with PTFE and HUV than with saphenous vein grafts. A significant difference in favor of the saphenous vein compared to the other prostheses was observed in patients operated for limb salvage; nonetheless, no significant difference was demonstrated among the three types of arterial substitutes in patients operated for claudication[59]. Weisel et al. demonstrated the superiority of saphenous vein compared to HUV and PTFE: at 3 years the patency rate was 78% for saphenous vein, 44% for HUV,

and 48% for PTFE in patients with low risk factors. On the other hand, in patients with high risk factors the patency rate was 66%, 32% and 28%, respectively[243]. Even worse results were obtained by Edwards & Mulherin with a patency rate of 24% for PTFE and 10% for HUV at 1 year compared to 82% for saphenous vein grafts[82].

Causes of graft failure

The causes of graft failure are multifactorial and complex. As an analogy of Virchow's triad, there are three broad classifications why grafts fail: inadequate surface, blood alterations and decreased flow through the graft.

Inadequate surface

Technical errors

Technical errors may create intimal flaps or severe narrowing at the anastomotic sites as well as ischemia of the artery adjacent to the anastomosis. The drying out of tissues, the use of traumatic vascular clamps and perhaps the suture material are other factors which may lead to thrombosis[72,151].

Thrombogenicity of the graft material

Current artificial materials are not truly blood compatible according to Mason's definition[158]. As a consequence of the interaction of blood with these materials, a layer of clot forms with the development of the neointima following organization. This neointima may be thrombogenic since it contains thromboplastins[211].

Development of anastomotic fibrous intimal hyperplasia

Anastomotic FIH is a common cause of intermediate and late failure of small diameter vascular prostheses[81,124,194]. The etiology is unknown but several factors have been implicated: 1) Surgical trauma, 2) hemodynamic factors, 3) compliance mismatch between artery and graft, 4) ischemia and 5) platelets[53,73,124,134,182]. Not only has FIH been the cause of failure in distal arterial reconstructions but in coronary bypass grafts and in arteriovenous fistulas[34,200]. It has also been described in association with several vascular substitutes including PTFE, HUV and saphenous vein grafts as well as in endarterectomized segments[58,81,134,181,200].

Blood abnormalities

Graft failure may be caused by pathological changes in the blood. Certain alterations in the blood composition during the postoperative period are known to increase the tendency of thrombosis. An increase in platelet adhesiveness has been demonstrated following surgery[16,112]. After an initial decrease in the fibrinogen content, a three to four-fold increase takes place during the postoperative period, at the same time a decrease in the fibrinolytic activity has been observed[157]. Another change which may have a deleterious consequence is a decrease in antithrombin III[2]. An increase in platelet adhesiveness and aggregability has been observed in arteriosclerotic patients[185]. Nevertheless, the effect of these pathophysiologic changes on graft patency has not been studied as such. The activation of the coagulation system is certainly of more significance than the elevation of the coagulation factors since the latter is not necessarily associated with intravascular thrombosis.

Decreased blood flow

Decreased blood flow through the graft secondary to a poor inflow or outflow or both is also a frequent cause of graft thrombosis[38]. Dean et al. noted that femoropopliteal grafts with flows less than 70 ml/min invariably thrombosed[68]. Patients with arteriosclerotic disease frequently have a high Hct[132], and an increase in blood viscosity[222]. Moreover, after trauma an increase in erythrocyte aggregation and blood viscosity has also been observed[17, 95].

Methods to improve outcome

Modification of graft surface

It is interesting that the creation of a vascular graft was a crude imitation of an artery, viewed merely as a conduit, ignoring the complex physiologic functions that the vessel wall provides[224]. Several characteristics of the fabrics such as porosity, durability and flexibility were early recognized as essential for a good outcome although there was limited knowledge of how they worked.

Porosity

Porosity is important for the incorporation of the grafts by the host tissue[40]. Pores allow the ingrowth of capillaries which will be necessary for nourishment of the neointima. This has been demonstrated in experimental animals, but in humans it is uncertain whether or not the incorporation of the graft is essential for the formation of a neointima[224].

Compliance

Lyman found that "nonthrombogenic" polymers occluded within a few days when they were tested in in vivo experiments. They felt that the mismatch in compliance between the host artery and the graft was the cause of the failure. By changing the elasticity of the grafts to approach that of normal arteries, the authors were able to show a marked improvement in the patency rates[155]. Walden et al. also demonstrated an increased patency rate in grafts with a compliance similar to that of the host vessel[240].

Flexibility

Flexibility can be achieved by crimping the graft or by making it elastic (compliant). A graft should be flexible for use in different anatomical areas e.g. crossing an articulation. Nevertheless, the flexibility and compliance may be lost after the graft is incorporated into the host tissues[224].

Durability

A graft should withstand the tissue reaction overtime; however, it should induce some degree of tissue reaction so that incorporation of the graft is allowed[224].

A comprehensive review is reported elsewhere[244].

Use of biologic tissue

Modified mandril-grown graft: It is a modification of Spark's mandril-grown graft. Silicone rods loosely wrapped with a polyester mesh are implanted in the subcutaneous tissue of sheep. Twelve weeks later, the implants are removed and treated with glutaraldehyde followed by rinsing with hydrogen peroxide 3%. The fibrocollagenous grafts are then ready for implantation[193]. These grafts have been implanted successfully in animals and humans; however,

other investigations encountered 100% failure when similar grafts were implanted in the carotid or femoral arteries of dogs[190].

Polyglactin 910 suture mesh graft: Polyglactin 910 suture mesh is an absorbable material which has been used as a framework for regeneration of arterial tissue in pigs. By the time the mesh is absorbed, a new vascular structure has been formed. This new vascular conduit has structural characteristics similar to the normal vessel on light and electronmicroscopy[28].

Endothelial seeding: In attempt to overcome the problem of limited regenerative capacity of the endothelium, seeding of arterial prostheses (dacron and PTFE) with endothelial cells has been carried out in experimental investigations. Seeded grafts have been tested in dogs successfully[104,105].

An ideal arterial substitute should: 1) have good blood compatibility, 2) allow adequate healing, therefore it would induce an appropriate tissue reaction so the graft is incorporated into the host tissue, 3) be flexible and compliant to accomodate to the different topographic demands, 4) be easy to handle, 5) be readily available; therefore easy to manufacture, and 6) be inexpensive.

Modification of blood

In theory, making the blood hypocoagulable or platelets less reactive should improve graft patency. Consequently, anticoagulant drugs such as heparin and warfarin have been used to prevent graft thrombosis for many years in experimental and clinical practice[88, 93,176,177]. Likewise, ASA or ASA combined with dipyridamole have frequently been given to patients undergoing revascularization procedures[83,162]. Dextran has also been administered in order to improve early patency, particularly in microsurgery[52,62, 138,189,248]. Discrepant results have been reported and will be dealt with more thoroughly in the discussion.

Modification of blood flow

Creations of arteriovenous fistulas have been performed in experimental and clinical practice to improve blood flow through the graft[25,175]. Their clinical application is rather limited, however. A sequential side-to-side anastomosis has also been reported to augment flow through the graft[129,130].

Hemodilution, by phlebotomy and/or dextran administration have also been employed to increase the peripheral blood flow[216].

PURPOSE

There is a great need for a better "off the shelf" vascular prosthesis and this is the main reason why this investigation was undertaken.

The aims of the present work were:

- To test vascular prostheses in high resistance and low flow conditions (I-IV) and at physiologic flow (IV, VI).
- To study the effect of heparin bonding on the thrombogenicity of biologic (HUV) and artificial materials (PTFE and PU) (II-III) and to study the stability of heparin bonding to HUV's over time (IV). In addition, to determine the effect of heparin on platelet activity in vivo (V).
- To produce the lesion FIH and to evaluate the effect of heparin bonding on its prevention (IV, VI).
- To study the effect of vascular grafting on the production of prostacyclin (PGI_2) and plasminogen activator (PA) (VII).
- To study the effect of ibuprofen on platelet activity in vivo and its effect on the prevention of graft thrombosis (I, VIII).

METHODS AND MATERIALS

More detailed information is given in the individual articles (I-VIII).

Graft performance

Carotid artery bypass model

Vascular prostheses of varying lengths but with a diameter from 4-6 mm were placed in the carotid arteries of dogs or sheep (II, VI and I, III, IV, VII, respectively). The anastomoses were made with extreme care. Running 6-0 or 7-0 polypropylene sutures were

used after placement of two everting stitches at each corner. Before clamping, the animal was heparinized and reversed with protamine after the last anastomosis was made (I, II). However, since protamine could possibly inactivate the bound heparin, the proximal and distal ends of the transected artery were flushed with 5 ml of sterile saline solution containing 4 IU/ml of heparin in subsequent experiments (III). In the intermediate and long term experiments heparin (1 mg/kg i.v.) was also given but reversal was not necessary (IV).

Comments

Dogs were used as the animal model in the first experiments. Notwithstanding, the dog appears not to be the ideal animal model for testing cardiovascular devices (National Institute of Health, Publication No 80-2185) because of its hyperplastic vascular response to stimulants and its very active hemostatic and fibrinolytic systems. Therefore, sheep were used in the following experiments since they have been reported to have coagulation parameters similar to man[230]. Sheep have other advantages: very accessible and long carotid arteries, easy to handle, readily available, adapt well to confinement and low cost.

Clamping of the artery during the performance of the anastomosis can lead to thrombus formation which would obviously lead to erroneous results. To avoid this problem, the protocol was originally designed to heparinize the animal before clamping followed by reversal with protamine after the last anastomosis was performed (I, II). Adequate reversal was determined with APTT assays. Whiffen, Young and Gott demonstrated that heparin activity was not affected by antagonists including protamine when heparin was bonded with a graphite-benzalkonium complex[245]. Nevertheless, it was still possible that protamine could inactivate the bonded heparin since different methods of heparinization were used; therefore, the protocol was modified (III) and instead, the ends of the artery were flushed with 5 ml of a saline solution containing 4 IU of heparin/ml. We are investigating the effect of protamine on bonded heparin at the present time.

Platelet deposition in vivo

Platelets were labelled with ^{51}Cr (II) or with ^{32}P (III)[3,247]. The radioactivity was recorded using an external detector at regu-

lar intervals for approximately 3 hours. When ^{51}Cr was used the radioactivity in the blood was determined and the ratio graft/blood ^{51}Cr activity was calculated (II). When ^{32}P was used the activity in the grafts was compared to that of carotid arteries (proximal to the grafts) and used as reference points. The ^{32}P activity in the platelet-rich plasma (PRP) was also determined.

Comments

^{51}Cr has frequently been used for labelling platelets. Two thirds of the ^{51}Cr incorporated into the platelets remains unbound in the cytoplasm; therefore, platelet stimulation is followed by a considerable release of the isotope[220]. ^{51}Cr is a γ-ray emitter. It was difficult to isolate the radioactivity of the graft from the activity coming from the rest of the animal body (II). This problem was avoided by using 20 cm lengths of grafts placed in a loop fashion and by isolating the exteriorized grafts with lead shields. However, the procedure was cumbersome and required long grafts and other factors such as turbulance could also influence the outcome. $^{32}Phosphorus$, on the other hand, is a β-ray emitter with short penetration. This allowed us to use a short graft (6 cm in length) and place it in the carotid arteries in a more "physiologic" manner. In addition, the regional distribution of platelet accumulation could be studied along the graft. $^{32}Phosporus$ is incorporated into the phospholipids of platelets and causes no change in the platelet survival[80]. The activity ranged from 75-88% in the PRP fraction and remained steady throughout the experiment.

Harvest of prosthesis

Patency: Patency was determined by measuring the blood flow with an electromagnetic flowmeter and by observation of bleeding from the divided artery distal to the graft.

Thrombus free surface (TFS): Upon removal, grafts were opened longitudinally, rinsed carefully in saline solution and the luminal surface photographed. The TFS was calculated by using a grid on the photographs.

Thrombus weight: Thrombus weight was measured at room temperature within 5 minutes after graft removal.

Light and electron microscopy: Representative sections from the anastomosis, graft and adjacent artery were fixed in buffered formaldehyde. Specimens were then stained with hematoxylin and eosin (H & H) and Masson Trichrome (MT). Similar sections were fixed in glutaraldehyde and subsequently prepared for scanning and transmission electron microscopy (SEM & TEM).

Comments

Undoubtedly, the ultimate goal is to maintain a graft patent; however, in a short term experiment a patent graft may be so filled with thrombus material that it would probably clot if the experiment lasted a bit longer. For this reason the evaluation of the thrombus-free surface is important since it gives more information in regards to the thrombogenicity of the vascular prosthesis[135, 210]. An example is given in figure 1.

Fig. 1. Nonheparinized polyurethane graft perfused at 25 ml/min for four hours. Graft was patent; however, the luminal surface was covered with thrombus material.

Frequently, grafts after removal show a film of proteinaceous material and this is not considered as thrombus.

The thrombogenicity of a graft material can be studied at different flow rates. The blood flow is regulated by placing an arterial occluder distal to the graft and is measured with an electromagnetic flowmeter. The thrombus threshold velocity (TTV) of a graft is then calculated. TTV refers to that flow at which 50% of the surface is covered by thrombus material[210]. Occlusion of grafts readily occurs when perfused with a flow below the TTV.

Methods to reduce thrombogenicity

Heparin bonding to biologic (HUV) grafts

Heparin and 50% ethylalcohol treatment (II, IV): HUV grafts were incubated in commercially available heparin (5000 IU/ml), for 12-24 hours, then flushed with saline and placed in ethylalcohol 50% for 24-72 hours (HAUV). These grafts were compared to nonheparinized HUV (NHUV) and heparinized HUV but without alcohol treatment (HsAUV). Grafts were irrigated with 3 liters of sterile saline solution before placement. In the acute experiments (II), the method of heparinization without alcohol treatment consisted of flushing the grafts with saline solution containing 10,000 IU of heparin/l [79]. In the other experiments (V), this method was modified and the grafts were then incubated in the heparin solution so that both HAUV's and HsAUV's were handled in the same manner. A similar amount of labelled heparin/surface area was observed between the two methods of heparinization: 65328 $\pm$ 19023 and 63790 $\pm$ 8661 CPM/cm^2 (M $\pm$ SD, n=3) for heparin flushing and heparin incubation respectively (unpublished data).

Heparin bonding to synthetic (PU and PTFE) grafts

Stabilized ionic bonding (III): Grafts are first treated with an aqueous solution of polyethyleneimine and glutaraldehyde, followed by treatment with a heparin solution. Second, the modified grafts are then treated with a solution containing colloidal particles composed of heparin and hexadecylamine hydrochloride and third, the ionic complex is stabilized with glutaraldehyde [147].

Covalent bonding (III): The first step is similar to that of ionic bonding of heparin. In the second step, the grafts are treated with dextran sulfate followed by treatment again with polyethyleneimine. The last step comprises the heparin bonding with a partly degraded heparin and this reaction is performed in water solution containing heparin and cyanoborohydride [145]. Commercially available heparin was also used in these two methods of heparinization (III).

Ibuprofen (I, VIII)

The effect of ibuprofen (isobutylphenyl propionic acid) on platelets was studied in rabbits. Platelet activity was assessed:

1) after inflicting a laser injury to arterioles in ear chambers and 2) after transection of arterioles or venules in the mesenteric microcirculation and determination of the platelet plug formation times. In addition, the effect of ibuprofen on thrombolysis was studied ex vivo using Chandler's model[1,46,126].

The effect of ibuprofen on the thrombogenicity of PTFE grafts was studied in sheep. Intravenous administration of ibuprofen was given at the time of the incision and thereafter every two hours.

Comments

The amount of heparin bound to a biologic surface - HUV in this case - depends on the concentration of heparin in the solution and to a lesser degree on the incubation time[122]. Glimelius, Busch and Höök showed that a progressive increase in the amount of bound heparin to endothelial cells from human umbilical veins occurred during the first 2-4 hours and then no further increase was observed. It was also demonstrated that the binding of heparin to endothelial cells reached a saturation point[98]. By incubating the grafts in heparin for at least 12 hours a saturation point should therefore be accomplished.

The artificial graft surface is modified by treatment with polyethyleneimine and glutaraldehyde; thus, a matrix containing secondary and tertiary amine groups is deposited on the surface.

For the stabilized ionic bonding of heparin, the negatively charged surface is transformed into a positively charged ionic group by treatment with hexadecylamine which diffuses into the surface. With the treatment with heparin, a zone of flocculation occurs close to the surface and an amine-heparin complex precipitates onto the surface. The glutaraldehyde treatment prevents the elution of heparin by cross-linking of the heparin molecules.

The treatment with dextran sulfate in the covalent bonding of heparin renders the surface negatively charged which allows the bonding of heparin by reductive amination.

The ear chamber microcirculation for studying the platelet function in vivo has the great advantage that the animals do not require anesthesia since it is known that the anesthetic agents may influence platelet function[22]. For the study of the formation of the hemostatic plug, the rabbits do require anesthesia and to mini-

mize this problem, the results after the drug administration were compared to those obtained before. Therefore, each animal served as its own control. These two methods have been studied extensively and reported elsewhere[6,18,20,166].

Thrombolysis was studied in thrombi made ex vivo. Such thrombi have gross and histologic characteristics similar to in vivo thrombi. The lysability was assessed by measuring the amount of radioactive fibrin split products released from the thrombus after incubation with plasmin or sodium chloride as well as by recording the thrombus weight before and after the administration of the drug. These methods have been described elsewhere[1,46,126].

Other factors which may influence graft performance

Heparin desorption (IV)

The stability of heparin bonding with and without alcohol treatment was investigated over time. Tritium labelled heparin was used to heparinize the HUV grafts. The half life of this isotope is 12.5 years. The radioactivity/cm^2 of luminal surface was determined before graft implantation and after graft removal. The percent of residual heparin was then calculated.

Comments

By using a labelled heparin solution, the grafts required a great deal of manipulation in order to avoid coating the external surface with the isotope. For the same reason, the grafts could not be completely filled with the heparin solution; therefore, it is possible that some areas were not in contact with the radioactive heparin. In order to measure the radioactivity of a β-ray emitter (tritium), the graft specimens had to be dissolved and placed in scintillation fluid, all this requiring several steps[192].

Effect of heparin on platelets (V)

The effect of heparin on platelet reactivity in vivo was studied in the ear chamber and mesenteric microcirculation of rabbits as described previously. Commercial heparin was compared with a low molecular weight heparin and a heparin analogue (sodium pentosan polysulfate). The drugs were given intravenously in comparable doses.

Comments

The study was performed in a blind fashion. The drugs were given intravenously to achieve similar levels of their concentration in plasma.

Anastomotic fibrous intimal hyperplasia (FIH) (IV, VI)

In attempt to produce the lesion, the following surgical procedures in the carotid arteries of dogs were performed: endarterectomy alone, endarterectomy plus autogenous vein patch angioplasty, interposition of PTFE grafts and endarterectomy plus PTFE angioplasty. Harvesting of grafts and/or operated carotid arteries was performed as previously described. Representative areas for light and electron microscopy were also obtained (VI). To demonstrate the effect of heparin bonding with or without alcohol on the prevention of FIH, HAUV and HsAUV grafts were interposed in the carotid arteries of sheep and removed 90 days later (IV).

Comments

Two different animal species were used, sheep (V) and dogs (VI) which make results difficult to compare. Nonetheless, it has been recently reported that FIH was the cause of occlusion of several vascular grafts including PTFE in sheep[42].

Prostacyclin (PGI_2) and plasminogen activator (PA) (VII)

Production of PGI_2 and plasminogen activator from the anastomoses and midgraft was determined by radioimmunoassay and a histochemical method, respectively.

Sections from arteries and veins were used as controls. For PGI_2 production, the specimens were incubated in Ringer's solution (pH 7.4) for 15 minutes and then transferred into another fresh solution and reincubated for another 15 minutes. The PGI_2 production/cm^2 of luminal surface was calculated[51,195]. The effect on collagen induced aggregation of some of the samples was investigated using an aggregometer.

For the PA activity, sections from the graft and host vessels were covered with plasminogen rich fibrin plate and incubated for

180 minutes[187,231]. Only the presence or absence of lytic activity was recorded at 30 min intervals.

Comments

The PGI_2 synthesis was determined by measuring the amount of 6-keto-$PGF_{1\alpha}$ in the incubation fluids. PGI_2 is very unstable with a half-life of 3 minutes at 37° C and a pH of 7.4[173,174]. Six-keto-$PGF_{1\alpha}$ is the final metabolite which is stable[186]. This assay is highly specific with a <2% cross reactivity with other prostaglandins (Data from New England Nuclear Instructions Manual). The presence of PGI_2 in the supernatant can be demonstrated by inhibition of platelet aggregation to collagen, arachidonate and ADP. This inhibition is prevented by pretreatment of the specimens with indomethacin or by boiling the incubation fluid[87,172]. The PGI_2 synthesis obtained from the graft and anastomoses was compared to that of autogenous arteries and veins since the normal values for sheep are not known. In addition, a great deal of variation has been reported within the same species[106]. Although the entire thickness of the vessel wall was incubated in the solution, it is known that most of the PGI_2 synthesis is produced by the intima[86,171].

Likewise, little information is available regarding the PA in ovine blood vessels; therefore, no effort was made to determine the magnitude of fibrinolytic activity but only whether or not lytic activity was present.

Experimental design

Graft performance

Short-term experiments

Grafts were observed from 3 (II) to 4 hours (I, III) and measurement of tagged platelet deposition was carried out for about 3 hours.

The effect of heparin and alcohol treatment of HUV was compared to NHUV placed in the contralateral arteries and to HsAUV grafts also placed in the contralateral carotid arteries (II). The blood flow in this group of experiments was restricted to 50 ml/min.

The thrombogenicity of PU and PTFE grafts was studied following stabilized ionic bonding of heparin. The new method of covalent bonding of heparin was also investigated in the PTFE's grafts (III). The blood flow in this group of experiments was reduced to 25 ml/min.

The effect of ibuprofen on the thrombogenicity of PTFE grafts was studied at 4 and at 24 hours and was compared to PTFE grafts from animals receiving no medication (I). The blood flow in these experiments was maintained at 50 ml/min.

Other factors which may influence graft performance

Intermediate and long-term experiments

HAUV's were compared with HsAUV's for 10 days with a blood flow of 50 ml/min and for 90 days with a physiologic flow. Heparin desorption as well as the production of PGI_2 and PA were investigated (IV).

The development of FIH was studied 60 days following placement of interposition grafts or endarterectomies with angioplasties in the carotid arteries of dogs (VI).

Platelet function in vivo

Ear chamber

Several drugs were tested including ibuprofen and commercial heparin. Two laser injuries were made before the drug administration and thereafter at 15, 60 and 90 min. The results were compared to the initial or predrug values (V, VIII).

Hemostatic plug formation time

Before and after the drug administration, 5 arterioles and 5 venules were transected (V, VIII).

Statistical analysis

Student's t test was used for paired values. When nonpaired observations were compared, nonparametric tests were used, e.g.Mann Whitney U or Wilcoxon tests.

To compare the effect of the drugs on the hemostatic plug formation times, the skew distribution was considered and the values were therefore rendered to a more normal distribution by logarith-

mic transformation. The significance of difference was then assessed by a nonparametric test.

The difference in the frequency of rebleedings was assessed by a rank sum test which avoids assumptions on the nature of the frequency distribution of the data.

The level of significance was set at $p<0.05$.

RESULTS

Detailed information is given in the individual articles.

Graft performance

Short term experiments

Heparin and alcohol treatment to biologic (HUV) grafts

Nonheparinized HUV grafts showed the highest thrombogenicity with a TFS of 13 $\pm$ 9%, 50% patency and the highest platelet accumulation. A marked improvement was observed after heparinization with an overall patency rate of 100%. However, the best results were obtained after the combination of heparin and alcohol, with these grafts showing the largest TFS (82 $\pm$5%) and the lowest ^{51}Cr platelet accumulation.

Heparin bonding to synthetic grafts

Stabilized ionic bonding of heparin to PU and PTFE grafts (III)

Stabilized ionic bonding of heparin diminished the early thrombogenicity of PU but not of PTFE grafts. The TFS for Hep-glut PU grafts was 57 $\pm$ 10 and only 1 out of 11 grafts thrombosed. The TFS for PU grafts was 27 $\pm$ 8 and the difference was statistically significant ($p=0.0207$).

In 5 experiments, ^{32}P platelet accumulation was determined in 5 Hep-glut PU and compared to PU grafts placed in the contralateral carotid arteries and it was found to be significantly reduced in the Hep-glut PU grafts. An uneven distribution of ^{32}P platelet deposition was observed with the distal anastomoses showing the highest ^{32}P platelet activity.

Covalent bonding of heparin to PTFE grafts (III)

Covalent bonding of heparin decreased the thrombogenicity of PTFE grafts significantly. Only 1 out of 8 grafts thrombosed and the TFS improved to 57% $\pm$ 11 from 27 $\pm$ 16.

^{32}P platelet accumulation in these grafts was also unevenly distributed with the distal anastomoses showing the highest activity.

Ibuprofen (I,VIII)

The administration of ibuprofen significantly reduced the tendency towards thrombus formation in PTFE grafts ($p<0.01$) at 4 hours. At 24 hours all PTFE grafts from animals which received ibuprofen were patent, whereas from untreated animals, all grafts thrombosed. At the same time ex vivo platelet aggregation was performed and only a moderate inhibition of ADP induced aggregation was observed. Consequently, the effect of ibuprofen on platelet function in vivo was studied (VIII). In doses of 10 mg/kg, ibuprofen did not appreciably influence the hemostatic plug formation times but in doses of 25 mg/kg, a prolongation in the primary hemostatic plug formation time - mainly platelet dependent - was noticed. The latter dose significantly inhibited platelet activity following a laser injury in arterioles. Even more interesting was the fact that ibuprofen significantly decreased the thrombus weight ex vivo and this effect seemed to be dose related. The greatest effect was observed with the largest dose (50 mg/kg i.v.) (VIII).

Other factors which may influence graft performance

Heparin desorption from HUV grafts

Intermediate term experiments (IV)

A trend towards a higher amount of residual heparin was observed in the heparinized grafts treated with alcohol. The difference was not statistically significant though (IV).

A total of 17 grafts were kept in place for 10 days. Three out of 6 HAUV's and 4 out of 6 HsAUV placed in the carotid arteries thrombosed.

A total of 5 grafts (3 HAUV and 2 HsAUV) placed in the jugular

veins also thrombosed. Thrombi in occluded grafts were not adhered to the luminal surface but to the anastomotic sites. Some of the patent grafts showed small proliferations in the luminal surface which correlated with a low concentration of residual heparin. These proliferations on histologic examination represented small mural thrombi mainly composed of fibrin and inflammatory cells. Small microthrombi were also seen at the anastomoses and had similar histologic characteristics.

Long-term experiments (IV)

A significantly higher amount of residual labelled heparin was found in the HAUV's compared to HsAUV's.

A total of 12 HUVG's (6 HAUV and 6 HsAUV) were kept in place for 90 days. One graft of each thrombosed. The HAUV showed clean luminal surfaces; however, two out ot 5 patent HsAUV's showed mural thrombi in the midportion of the grafts. These findings were confirmed histologically.

Effect of heparin on platelets in vivo (V)

Commercial heparin was found to prolong the hemostatic plug formation times in doses of 60 IU/kg i.v. Heparin also induced an increase in platelet reactivity after a laser injury to the arterioles of the ear chamber in rabbits (V). Low MW heparin did not affect the formation of the hemostatic plug; nevertheless, its effect on platelet reactivity in vivo was similar to that of commercial heparin.

Anastomotic fibrous intimal hyperplasia (IV-VI)

Anastomotic FIH was not observed following endarterectomy alone or endarterectomy with vein angioplasty. In contrast, FIH developed after placement of PTFE interposition grafts or endarterectomies with PTFE angioplasties. Approximately 50% of the grafts and PTFE angioplasties thrombosed and the cause in these instances was FIH. In at least half of the patent ones, the lesion significantly narrowed the luminal diameter (50% or more). FIH consisted mainly of smooth muscle cells, migrating from the media into the intima through a fragmented elastic membrane (VI).

FIH was also investigated in the HAUV's and HsAUV's. In some of the anastomotic areas, particularly in the HsAUV's, a shiny, slightly raised, whitish lesion was observed by gross inspection.

These lesions on light microscopy proved to be early FIH. While the normal intima contains one or two layers of cells, these particular areas showed intima with 5-6 layers of cells (IV). These lesions were very small compared to the FIH from the other experiments (VI), and in none of the grafts was the lumen of the anastomoses compromised. Although on light microscopy, the anastomoses appeared to be endothelialized, several pathologic changes were observed on SEM. Some of the endothelial cells were large and pleomorphic. A change in their orientation was also observed and in some cases platelet and leucocyte adhesion was observed in areas denuded of endothelial cells (IV-VII).

In contrast, with PTFE interposition grafts or endarterectomies with PTFE angioplasties, FIH was present in 100% of the cases and was the cause of thrombosis in several (VI) (Fig. 2).

Fig. 2. Photomicrograph from proximal anastomosis of a PTFE graft showing marked narrowing of the lumen secondary to fibrous intimal hyperplasia. Graft was removed four months later.

Prostacyclin (PGI_2) and plasminogen activator (PA)

The incubation fluid was found to inhibit collagen-induced aggregation, an effect which was abolished by boiling the specimens.

Intermediate term experiments (VI)

The results of PGI_2 from the anastomoses and midgraft of the HUV grafts placed in the carotid arteries and jugular veins are shown in figures 3 and 4. In these two groups the PGI_2 synthesis from the midgraft was lesser than the control vessels. From the grafts placed in the carotid arteries, the PGI_2 synthesis was higher than that of control arteries; however, when these grafts were subgrouped in patent and thrombosed, the occluded grafts showed a significantly higher PIG_2 production from the anastomoses (Fig. 5). In contrast, the anastomotic PGI_2 production from the occluded

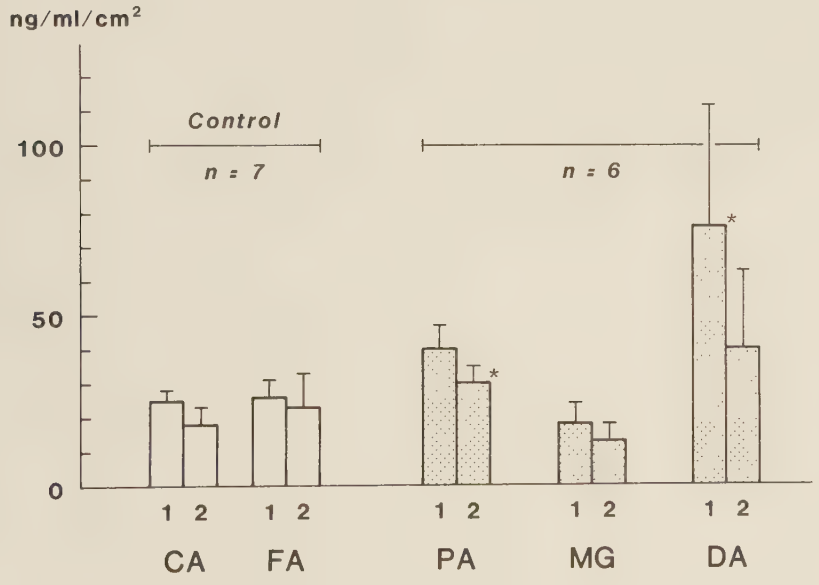

Fig. 3. Prostacyclin (PGI_2) synthesis from anastomotic sites and midgraft of HUV grafts placed in the carotid arteries of sheep and removed 10 days later ($M \pm SE$). CA=carotid arteries, FA=femoral arteries, PA=proximal anastomoses, MG=midgraft, DA=distal anastomoses, 1=first incubation period and 2=second incubation period. p=0.069 vs control carotid and femoral arteries.

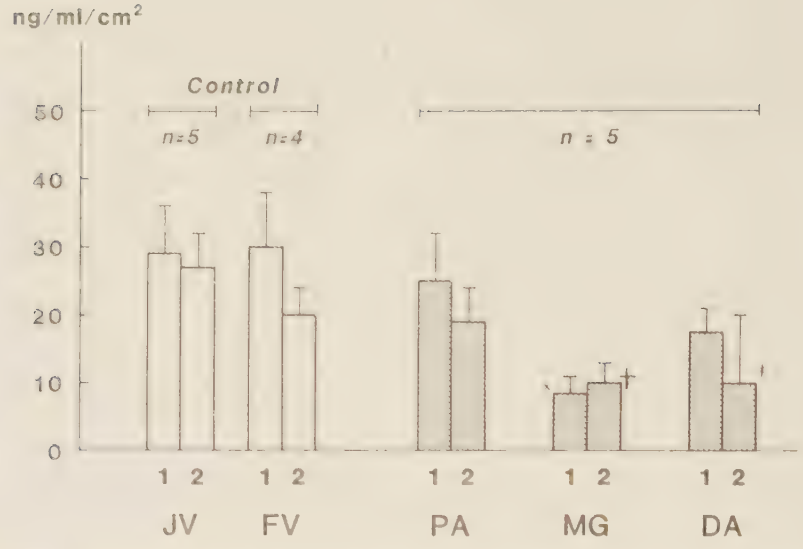

Fig. 4. PGI$_2$ synthesis from anastomotic sites and midgrafts of nat grafts placed in the jugular veins of sheep and removed 10 days later (M±SE). JV=jugular veins, FV=femoral veins. For code, see Fig. 1.
* p=0.016 vs control jugular and femoral veins; † p=0.016 vs jugular veins, p=0.033 vs femoral veins; ‡ p=0.008 vs jugular veins, p=0.032 vs femoral veins.

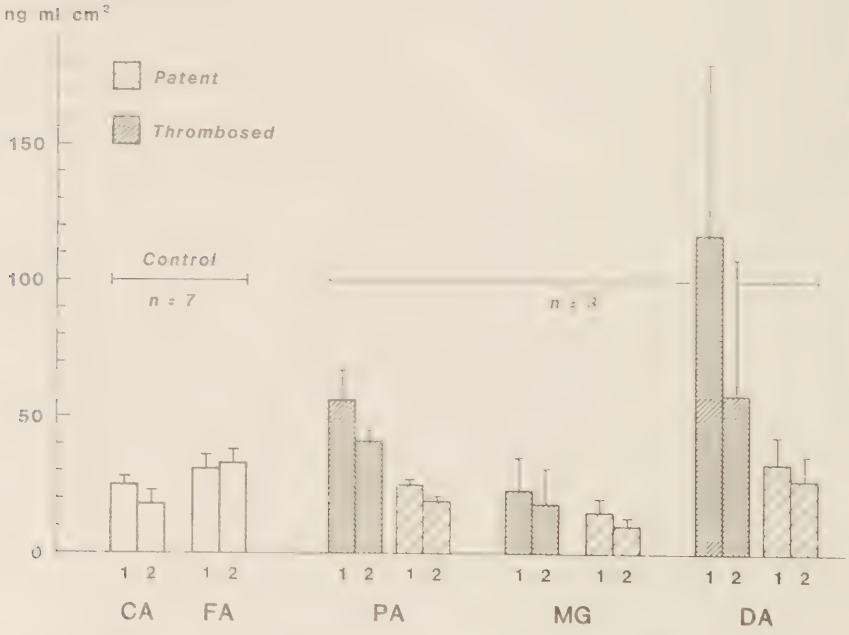

Fig. 5. Anastomotic and midgraft PGI$_2$ synthesis from patent and trombosed grafts placed in the carotid arteries of sheep and removed 10 days later (M±SE). For code, see Fig. 1.

grafts placed in the jugular veins was similar to or slightly lower than that of control veins (Fig. 4).

A total of 11 grafts were tested for PA and all of them showed lytic activity in the adventitia but in none was there intimal PA activity.

Long-term experiments (VII)

The PGI_2 synthesis from the HUV grafts removed 90 days later is summarized in figure 6. The PGI_2 production from midgraft was also less than that of control arteries.

Nine HUV grafts were tested for PA activity and all of them showed adventitial lytic activity and 6 out 9 PA activity.

For the histologic findings the reader is referred to the corresponding article (VII).

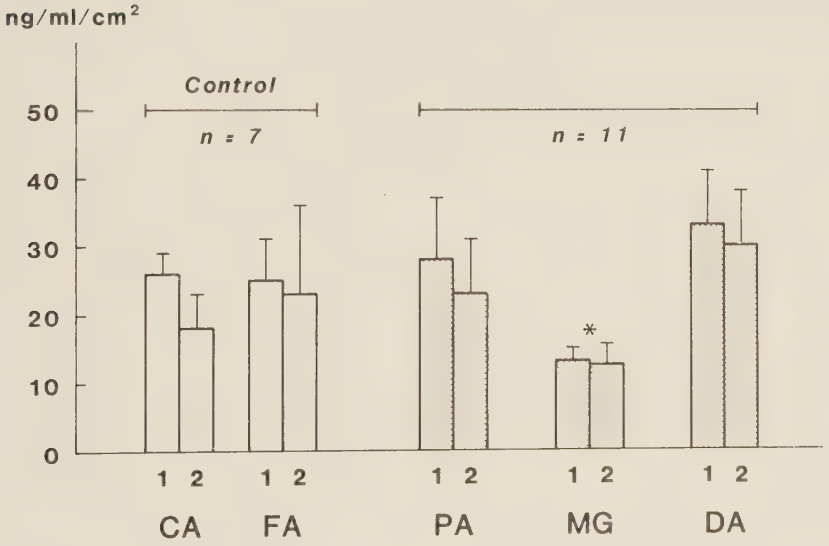

Fig. 6. Anastomotic and midgraft PGI_2 synthesis from HUV grafts placed in the carotid arteries of sheep and removed 90 days later (M±SEM). For code, see Fig. 1.

** p=0.082 vs control carotid and femoral arteries.*

GENERAL DISCUSSION

The need for methods to prevent graft occlusion is enormous. Poor performance of small diameter vascular prostheses (<6 mm) is a frequent occurrence in low flow and high resistance locations. Thus far, the best arterial substitute is the autogenous saphenous vein[67,74,76,82,243,250]; however, in 15 to 30% of the cases, the vein cannot be used because of small size, multiple branches, gross abnormalities or because it had been removed previously[151, 225,226]

Glutaraldehyde-tanned HUV and PTFE grafts are the most common vascular prostheses used for revascularization for lower limb ischemia. They have been successfully used by some[39,64,108,235], but others have reported very discouraging results[82,250]. Although some improvement has been achieved by modifying the physicochemical characteristics of the graft[244], it appears that a concomitant antithrombotic treatment or binding of a pharmacologic agent to the surface is necessary in order to reduce the early thrombogenicity of the graft material and the late development of FIH[38].

Heparin bonding to biologic (HUV) grafts

Björck et al. recently demonstrated that fresh human umbilical veins adsorb and inactivate thrombin and that this particular property was lost when the veins were tanned with glutaraldehyde. However, tanned human umbilical veins regained the property to inactivate the adsorbed thrombin after being treated with heparin and ethyl alcohol 50%. Heparinization alone did not seem to have this particular effect although the sample size was too small for statistical evaluation[23]. Moreover, for the use of HUV , it is recommended to irrigate the conduit with 6 liters of saline containing 10.000 IU of heparin/l although the potential benefit of heparin in this solution has not been proved[79]. The purpose of the irrigation is to eliminate the glutaraldehyde residues which appear to have a thrombogenic effect. Therefore, it was worthy first to evaluate whether or not heparin had a beneficial effect in the irrigant solution and second to compare the effect of the two types of heparinization (with and without alcohol) on the

thrombogenicity and prevention of FIH. A few fresh human umbilical veins were placed in the carotid arteries of sheep but all of them thrombosed (unpublished data). Tanned human umbilical vein grafts treated with heparin and alcohol showed the lowest thrombogenicity. All HsAUV's were patent but TFS was significantly reduced compared to HAUV's. The worst results were obtained with the NHUV grafts. Hence, the addition of heparin to the irrigant solution definitely had a beneficial effect. No difference was demonstrated between HAUV and HsAUV in the intermediate and long term experiments, although the HsAUV frequently showed small mural thrombi in the midgrafts, but the significance of this finding is unknown. The alcohol, though, delayed the heparin desorption. Then, why was there a difference in the short term experiments but not in the intermediate and long term experiments? There is not a clear answer, but several factors could have influenced the results. In the acute experiments, protamine was given to reverse the heparin administered during the performance of the anastomoses. Perhaps the alcohol induced conformational changes in the heparin molecule rendering it less susceptible to protamine inactivation whereas the heparin without the alcohol was readily inactivated. This could explain why there was a significant difference in the acute experiments and not in the intermediate and long term experiments since protamine was not given. This explanation remains to be proved, however. In the acute experiments, there was also a constant control of the blood flow and other factors such as kinking or external compression could be prevented. In the intermediate term experiments, a reduction in the cardiac output especially during the postoperative period could have had deleterious consequences since the blood flow through the graft was already reduced.

In the chronic experiments, the results were very good for both HAUV and HsAUV's in regards to patency rate. Some of the grafts showed early lesions of FIH but in none of them was the lumen compromised. This is in contrast to the poor results obtained by Oblath et al. using HUV grafts. In 6 out of 7 grafts which occluded the cause was FIH[181].

All HUV's placed in the jugular veins failed. The problems with venous grafting constitute a different issue, so they will not be discussed any further.

Heparin bonding to synthetic grafts (PU and PTFE)

The next step was to heparinize polymers and use them as arterial substitutes. Successful heparinization of a hydrophobic polymer (polyethylene catheter) has been achieved by Larsson et al.[147]. The increased blood compatibility of this particular catheter has been demonstrated in in vitro and in vivo experiments[146-149]. Two materials were heparinized, PTFE and PU and two methods of heparinization were investigated. Stabilized ionic bonding of heparin to PU grafts significantly reduced their acute thrombogenicity compared to untreated grafts but not to PTFE grafts. A covalent bonding of heparin onto PTFE grafts met with excellent results though. The different results otained after stabilized ionic bonding of heparin onto PU and PTFE are probably related to variation in the physicochemical characteristics of these polymers.

In 1963, ionic bonding of heparin was introduced by Gott et al. who demonstrated that a graphite-benzalkonium surface is thromboresistant[101]. Due to the limitations of obtaining an adequate layer of collodial graphite in several substrates and the rapid loss of heparin[141], multiple procedures to bind heparin to other cationic groups have been performed but with limited success basically because of the poor stability of the heparin bonding[146]. In order to prevent heparin desorption, the heparin bonding was stabilized with glutaraldehyde. In experiments in vivo, after an initial loss of 12% no further loss was noted for 7 days[148].

Covalent bonding of heparin is an alternative procedure to prevent heparin desorption. Unfortunately, surfaces coated with covalently bonded heparin appear to be less platelet compatible[152], probably due to the decrease in the number of biologically active sites available. The covalent bonding in these experiments has been modified so that one end of the heparin molecule is bonded to the surface by means of a residue. This type of conformation allows the active site or sites of the heparin molecule to be free. Although it is too early to say, this method of heparinization seems promising. At any rate, heparin-coated vascular prostheses were significantly less thrombogenic than untreated surfaces.

A consistent finding in the occluded heparinized grafts was the fact that the thrombus material was not adhered to the luminal

surface but firmly attached to the anastomotic sites. Moreover, during the determination of platelet accumulation in vivo, it became apparent that a significant increase in platelet deposition occurred in the anastomosis, particularly in the distal anastomosis and similar findings were recently reported by others[47].

In vivo tests were performed to evaluate the effect of heparin on platelets because of the conflicting reports in the literature in that respect[183,209,229]. Heparin seemed to increase platelet activity; however, there was a decreased platelet accumulation in the heparinized surface compared to the untreated surface. These results are in accord with the findings reported by Lindsay et al. who showed a platelet antagonistic effect with heparin in solution and a favorable effect with bonded heparin[153].

Anastomotic fibrous intimal hyperplasia

Anastomotic fibrous intimal hyperplasia has received little attention as a cause of graft failure although it is a common cause of occlusion of medium and small size arterial prostheses in patients and in experimental animals[73].

Anastomotic FIH was induced by the insertion of PTFE interposition grafts and by endarterectomies with PTFE angioplasties. Compliance mismatch and altered hemodynamic conditions may have been responsible for the development of FIH. Platelets did not seem to play a role since the endothelium overlying the lesion was intact. Early FIH was also observed in some of the HUV grafts but in none of the cases was the luminal diameter compromised. Anastomotic FIH was observed more frequently in the HsAUV than in the HAUV's and this is probably explained by the presence of heparin (in the HAUV) since heparin has been reported to have en antimitogenic effect[191]. Because the grafts were removed three months later, it remains unknown whether heparin had a preventive effect or just delayed the progression of the lesion. However, and in contrast to the other experiments, the endothelium overlying the anastomoses was abnormal and the difference between the two groups of experiments is probably related to species variation since the regenerative capacity of canine endothelium is very rapid[241].

Prostacyclin (PGI_2) and plasminogen activator (PA)

Since it is nothing new that the anastomosis is a source of thrombus formation[41], an attempt was made to study the effect of vascular grafting on the production of PGI_2 and PA.

Interesting findings were obtained in the intermediate term experiments. In the occluded grafts placed in the carotid arteries, it is unknown whether the PGI_2 synthesis was high or low before thrombosis occurred. The former would indicate that the stimulus for thrombus formation overcame the PGI_2 protective effect. On the other hand, the latter would mean that thrombosis ensued because of the low PGI_2 synthesis and the presence of the thrombus would then stimulate PGI_2 production. The rationale becomes more complex when evaluating the findings from the anastomoses of the HUV grafts placed in the jugular veins. These grafts were thrombosed and the anastomotic PGI_2 synthesis was similar to that of control veins. One can speculate that the presence of platelets, being the main component of arterial thrombi, would stimulate PGI_2 synthesis from the arterial wall; whereas, in the venous system this stimulation would not occur because platelets are not the main component in venous thrombi. This would explain why the PGI_2 synthesis was high in the anastomoses of thrombosed grafts placed in the carotid arteries and normal from the anastomoses of occluded grafts interposed in the jugular veins.

In the long term experiments, the PGI_2 synthesis from anastomotic sites was similar to that of control arteries. However, it was interesting to find that some of the anastomoses which appeared endothelialized on light microscopy, showed areas with deformed endothelial cells or areas of endothelial denudation accompanied by adhesion of leukocytes and platelets i.e. stimulus for thrombus formation. It is intriguing that the PGI_2 synthesis was not elevated. The PGI_2 synthesis was not determined by layers. If we had been able to show that the abnormal intima produced a diminished amount of PGI_2 and that the PGI_2 came from the smooth muscle cells; it would have strongly suggested that the cause for smooth muscle proliferation was secondary to a persistent stimulus for thrombus formation at the anastomotic level. It is possible that leukocytes and platelets are responsible in the pathogenesis of this lesion. Platelets are known to possess a mitogenic factor and infiltration with leukocytes is frequently seen at the anastomotic site from

the early postoperative period. Platelets may not be the only responsible factor, however, since in the dog experiments (VI) an intact endothelium was seen overlying the anastomoses. It is more likely that several factors may be involved in the pathogenesis of FIH. Adverse factors at the level of the anastomoses e.g. hemodynamic factors, compliance mismatch between artery and graft, operative trauma may be responsible for the development of FIH by inflicting a persistent trauma to the vascular wall.

The production of PGI_2 by the neointima in the midgraft was always less than that of control vessels and similar results have been reported elsewhere[51,87].

There are several reports in the literature regarding the PA activity in arteries, veins and vein grafts[77,187,249]. Veins have been shown to have more PA than arteries[77,232], and similar findings were observed in the present investigation although the magnitude of lysis was not recorded.

The presence of PA in the adventitia in arteries and veins has been a subject of great discussion. Still debatable, it appears that the PA's are transported into the lumen via the vasa vasorum; although, vasa vasorum has not been observed in the intimal layer.

All HUVG's showed PA activity in the adventitia and two-thirds of the long-term implanted HUVG's also showed PA's in the neointima. Vasa vasorum was seen in the adventitia but not in the neointima. In the long-term experiments, all grafts remained patent; therefore, the potential benefit of neointimal PA activity on patency could not be demonstrated nor could it be correlated with the development of FIH. Yao et al. found that occluded Dacron grafts had no PA whereas in 3 out of 5 patent grafts PA was demonstrated[249]. In contrast to our results, Puckett et al. already observed PA in the developing neointima of knitted Dacron grafts 24 hours after surgery[196].

The presence of the PA in the adventitia of HUV's is probably not significant. The healing process with the neoformation of capillaries and the leukocytes in the tissue reaction to the polyester mesh used in the HUV's are most likely the source of PA[142]. If the PA can be transported into the lumen of the graft is unknown; nevertheless, the presence of PA in the neointima may be of significance.

Adjuvant antithrombotic therapy

The usefulness of antiplatelet or anticoagulant drugs on postoperative vascular patency has been investigated for over 40 years[11, 29,63,114,138,176,177]. Murray and Best demonstrated in 1948 the beneficial effect of heparin on prevention of thrombosis in experimental vascular surgery[177]. Soon after, it was successfully used in patients undergoing vascular surgery[176]. Since then heparin has been used intraoperatively in order to prevent thrombosis during vascular clamping in revascularization procedures[12,116]. However, heparin has been reported to induce platelet aggregation in vivo leading to thrombocytopenia as well as being the cause of arterial embolization[10,133]. The variable effects of heparin on platelets probably depend on the heterogeneity of the heparin molecule[24,49,103,119]. Its fractionation has led to new heparin fragments with more selective action; thus, low molecular weight (LMW) heparin has been shown to have a high antifactor X and a low antithrombin activity and the reverse has been observed with high molecular fractions[5,144]. Furthermore, Salzman demonstrated that a high molecular weight heparin with low or high affinity for antithrombin III increased platelet aggregation in vitro and a similar effect was observed with a LMW heparin with low antithrombin III affinity. However, a lesser effect on platelet aggregation was noted with the LMW heparin with high antithrombin III affinity [209]. In studies of platelet function in vivo, both LMW and commercial heparin increased platelet reactivity (V). Nevertheless, heparinized surfaces were observed to decrease platelet deposition (II-III) and similar reports have been reported elsewhere[143,147]. These findings are in agreement with those of Lindsay et al. who demonstrated an antagonistic effect of heparin on platelets which depended on heparin being in solution or bonded to surfaces[153].

There are also several reports about the effect of antiplatelet drugs on normal and injured intima as well as on endothelial cell cultures[36,55,61,71,93]. Clagett et al. investigated the effect of ASA, dipyridamole and low molecular weight dextran on patency of autogenous vein grafts placed in the iliac arteries of dogs. All these drugs decreased thrombus formation but ASA was more effective than dipyridamole and low molecular weight dextran[52].

Hoepp, DeWeese and Elbadawi studied the effect of steroids and

azathioprine on the development of FIH following placement of Dacron bypass grafts in the femoral arteries of dogs. One hundred % patency rate was found in the azathioprine group and correlated well with the absence of FIH whereas steroids did not have any significant effect. A more organized intimalization in the immunosuppressed group was observed. Using the same animal model[117], Oblath et al. achieved a 100% patency in Dacron® and PTFE grafts after treatment with ASA and dipyridamole. Grafts were removed 4 months later and no evidence of FIH was noticed. In contrast, a 60% patency rate was found in the untreated group and FIH was found in one third of the grafts[182]. McCann et al. did not obtain such good results using ASA and dipyridamole in rhesus monkeys[163]. It can be argued that in McCann's study the dose of ASA falls into a large dose category whereas in Oblath's into the small dose category considering the weight of the animals. Hagen et al. also demonstrated a significant reduction of anastomotic intimal hyperplasia with ASA and dipyridamole in nonhuman primates [109]. A decrease in intimal thickening in dog coronary bypass vein grafts with dipyridamole and aspirin was reported by Metke et al.[167].

However, there are reports that ASA had no effect on platelet adhesion[71], or that it may actually enhance platelet adhesion due to the inhibition of prostacyclin by the intima[36]. This phenomenon has also been shown in in vitro experiments[61]. Failure to prevent myointimal proliferation in injured carotid arteries of rats by ASA and other antiplatelet drugs was reported by Clowes and Kannovsky[55]. In a recent report, Schaub, Rawlings and Keith showed that the administration of ASA to dogs with chronically damaged pulmonary arteries had no effect on platelet adhesion after 4 days of injury, but after 30 days of treatment ASA significantly reduced platelet adhesion compared to the untreated animals. The authors suggested that the long-term administration of ASA probably induces changes in the platelet membrane which would impede platelet adhesion independently of the prostaglandin mechanism[215].

Several clinical studies have been published evaluating the effect of antithrombotic therapy on graft patency although the majority of these trials have been performed on patients with coronary artery bypass grafts using the autogenous saphenous vein. The results are conflicting, with some reporting an improvement[15,

35,162), and others reporting no effect(138,164,188).

Harker et al. demonstrated that in patients who underwent placement of vascular prostheses, the platelet survival time decreased to as low as 3-4 days from 9 days in the controls and the platelet turnover increased 2.5 to 3 fold over the initial values. After the first month following surgery the platelet survival and turnover progressively began to return to normal values, a process which took almost a year. They were able to reproduce the same phenomenon in baboons undergoing bypass grafting and the changes correlated well with the degree of endothelialization. Furthermore, by administering ASA and dipyridamole within the first month after surgery, the abnormal platelet survival and turnover was stopped. As a matter of fact, in two patients the platelet survival returned to normal levels with the administration of Dextran 40 and when the latter was discontinued the platelet survival again decreased to approximately 4 days(113). A significant reduction in late graft occlusion in patients who received ASA was reported by Ehresman, Alemany and Loew(83). Evans and Irvine found no improvement with warfarin in patients who underwent femoropopliteal bypass(88). Brands also reported no effect(30), and likewise, Blakely and Pogoniler reported no improvement with sulfinpyrazone in patients with femoropopliteal vein or prosthesis bypass(26).

The trial design, the type of drug and dose, the introduction of antithrombotic treatment in relation to surgery, the different patient population in addition to different methodology and animal model are just a few of several factors which can explain the varying results. ASA offers a special problem since its effect seems to be dose related.

ASA acetylates the enzyme cyclo-oxygenase(201), and in the platelets this reaction is irreversible since platelets cannot synthesize the enzyme. Therefore, inhibition of TxA_2 persists for their whole life span. The same reaction occurs in the endothelial cells, but as opposed to the platelets, synthesis of the enzyme cyclo-oxygenase - and hence PGI_2 production - occurs within hours(172, 174).

A small dose of ASA prolongs the bleeding time but a large dose does not(184). This particular dose-related effect on the cyclo-oxy-

genase needs reassessment because of conflicting reports in the literature. Baezinger noted that endothelial cyclo-oxygenase was much less sensitive to ASA than platelet cyclo-oxygenase[8]. A small dose of ASA would therefore inhibit the platelet but not the vascular enzyme. Nonetheless, Jaffe and Weksler reported that the endothelial cyclo-oxygenase was more sensitive to ASA than the platelet counterpart but the endothelial cells could replace the enzyme within hours[127]. Preston et al., on the other hand, found very little difference in the inhibition of platelet or intimal cyclo-oxygenase with doses of 150 and 300 mg of ASA in normal individuals[195]. The problem is compounded even further by the fact that the effect of ASA seems to depend on the age and sex of the patient[131]. Because of the serious problem of increased intraoperative bleeding with ASA[233], the effect on ibuprofen on graft thrombogenicity was studied in short-term experiments. Ibuprofen was found to significantly increase the TFS and patency in PTFE grafts. In a recent investigation, 100% patency at 30 days was obtained in 1 mm PTFE grafts placed in the abdominal aorta of rats with the administration of ibuprofen compared to 10% patency in the group receiving no medication[54]. Moreover, the administration of ibuprofen was noted to decrease platelet deposition on PTFE grafts significantly[140]. In addition, ibuprofen was found to inhibit platelet activity and to decrease the thrombus weight of thrombi ex vivo. This effect is probably caused by the structural change induced by ibuprofen, since formation of the head no longer occurred and the platelets were more widely distributed throughout the specimen. Ibuprofen is a non-steroidal anti-inflammatory drug, but unlike ASA, its effect on platelet cyclo-oxygenase is reversible[165,197]. Although on gross inspection increased bleeding was not noticed during the surgical procedure, ibuprofen in a dose of 25 mg/kg was found to interfere with the hemostatic plug formation time. In clinical trials ibuprofen has been demonstrated to have fewer side effects than ASA[44,75,123].

CONCLUSIONS

In the present work, we have principally dealt with one aspect: the blood compatibility of vascular prostheses and from the results the following conclusions may be drawn:

- The carotid artery interposition model was an adequate method to evaluate the thrombogenicity (platelets and plasma coagulation compatibility) of arterial substitutes in conditions of high resistance and low flow. It also allowed the study of factors which may play a role in the pathogenesis of FIH.

- Proper selection of methods of heparin bonding increased platelet and enzymatic coagulation compatibility of HUV, PU and PTFE grafts. Heparin and alcohol treatment significantly decreased the thrombogenicity of HUV compared to HsAUV and NHUV grafts. Stabilized ionic bonding and covalent bonding of heparin were effective in diminishing the thrombogenic properties of PU and PTFE grafts, respectively. Stabilized ionic bonding of heparin onto PTFE was ineffective.

  Thrombi on heparinized grafts were not adhered to the luminal surface but to the anastomotic sites; therefore, the anastomoses appear to be a strong stimulus for thrombus formation.

  The alcohol treatment following heparinization of HUV significantly delayed the heparin desorption.

  Heparin altered the formation of the platelet plug and increased the reactivity of platelets in vivo.

- The interposition of a PTFE graft or endarterectomy with PTFE patch angioplasty leads to the development of FIH. Heparin bonding did not prevent FIH but appeared to limit its progression.

- A variable PGI_2 production from anastomotic sites occurred and seemed to depend on graft patency and grafting site. HUV grafts produced PGI_2 but less than normal vessels.

  The neointima of HUV grafts also gained the ability to produce PA later in time and PA was always found in the adventitia of these grafts.

- Ibuprofen decreased platelet activity in vivo and thrombus weight ex vivo and improved the performance of PTFE grafts in conditions of high resistance and low flow.

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1

TESTING AND TREATMENT OF ARTERIAL GRAFT THROMBOSIS

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Testing and Treatment of Arterial Graft Thrombosis

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Testing and Treatment of Arterial Graft Thrombosis

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Thrombosis has been implicated as a prime cause of early arterial bypass graft failure [*1–3*]. A significant factor involved in the failure of these arterial grafts is the thrombogenicity of the graft material. From the standpoint of long-term prosthetic arterial graft patency, it appears desirable to reduce the thrombogenicity of the prostheses to allow the time needed for tissue ingrowth and healing of the luminal surface. When prosthetic healing is allowed to occur in an orderly fashion after a reduction in thrombogenicity, further antithrombotic regimens are not needed [*4,5*]. Thromboembolic complications secondary to the foreign body-blood interface reaction occur at a reduced rate in healed prostheses. Healing also appears to decrease the incidence of neointimal fibrous hyperplasia, a frequent cause of medium-term occlusion in smaller arterial bypasses [*6*].

Recently an in vivo thrombogenicity test simulating the acute conditions under which arterial bypass grafts are inserted was proposed by Sauvage et al [*7*]. Arterial graft material is interposed in the carotid artery position of an experimental animal. Different flow rates through the graft are maintained for varying periods of time, and the luminal surface of the graft is then examined. Results of these experiments indicate that arterial grafts with small amounts of luminal thrombus at low flow rates should have higher patency as chronic implants.

Although this test shows promise as a relative determination of arterial graft thrombogenicity, its use in predicting long-term patency of arterial prostheses with a small diameter (6 mm or less internal diameter) appears limited [*8*]. It appears unlikely that existing small diameter arterial prostheses will maintain long-term patency without antithrombotic therapy [*8,9*]. The frequency and type of antithrombotic therapy that may be useful remains controversial and the ability to evaluate the effectiveness of varying antithrombotic regimens has become important. Using a modification of the Sauvage arterial graft thrombogenicity determination, we studied the antithrombotic effect of ibuprofen (Motrin®, Upjohn) on the thrombogenicity and patency of small diameter prosthetic arterial grafts. Ibuprofen, commonly used for its anti-inflammatory and analgesic effects in musculoskeletal disorders, was chosen because of specific antithromboxane properties which inhibit platelet aggregation but do not affect the ability of the host vessel to resist thrombosis.

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Material and Methods

Sheep carotid interposition model: Mature, wether sheep less than 18 months old of Colombian breed were used exclusively in this study. The animals weighed 40 to 45 kg and were maintained in corrals under strict veterinary supervision on ad lib exercise routines. Diets consisted of alfalfa pellets, salt and water.

The sheep carotid interposition model was used because we have determined that its coagulation parameters and platelet function resemble those of man [*10*]. These parameters remain consistent under conditions of normal activity, inhalation anesthesia and vascular surgery of the type used in this experiment. Using this model, 5 cm lengths of commercially obtained expanded polytetrafluoroethylene arterial grafts (Gore-Tex®, William Gore and Associates) with an internal diameter of 4 mm were sewn end-to-end in the carotid arterial position using running 6-0 polypropylene suture (Prolene®, Ethicon). A 4 cm segment of artery was removed. Anesthesia was maintained using nitrous oxide, halothane and oxygen mixtures endotracheally. Using a proximal, external electromagnetic flow probe and distal arterial occluder as described by Sauvage, the polytetrafluoroethylene grafts were perfused at 25, 50, 75 cc/min and 100 cc/min for 4 hours [*7*]. Grafts were then removed and opened promptly, and high resolution photographs were made of the luminal surface and adjacent artery. The amount of thrombus-free surface on each luminal surface was then calculated using a grid and plotted for each flow rate. Totally occluded grafts were considered to have a thrombus-free surface of 0. This de-

TABLE I Thrombus-Free Surface (TFS) of Expanded Polytetrafluoroethylene in 4 Hour Flow Study

Flow Rate (cc/min)	Percent TFS (mean ± standard error of the mean)
25	22.0 ± 21.6
50	50.4 ± 20.8
75	78.3 ± 17.9
100	80.4 ± 15.2

termination was repeated at each flow rate 5 times and averaged. Using 5 cm lengths of autogenous carotid artery and jugular vein, these determinations were repeated in the same manner. The average was then plotted against the flow rate for each of the three grafts employed. From the resultant curves, the thrombotic threshold velocity for each material was derived as the flow below which the thrombus-free surface falls to 50 percent. It has been noted previously that a prosthetic arterial graft may be expected to occlude readily when perfused at flows below its thrombotic threshold velocity.

Ibuprofen administration and effect on thrombus-free surface: As 50 cc/min appeared to be a critical flow rate for expanded polytetrafluoroethylene, this rate was used for the second part of this experiment employing ibuprofen administered intravenously. Two groups of five sheep each had polytetrafluoroethylene grafts placed bilaterally in the carotid arterial position and flows were adjusted to 50 cc/min. On one side, grafts were removed at 4 hours and on the other at 24 hours. Thrombus-free surface was determined as before.

One group of five animals received ibuprofen intravenously at the following dose schedule measured from the time of the initial incision: first dose, 13 mg/kg, incision; second dose, 6.5 mg/kg, 2 hours later; third dose, 13 mg/kg, 2 hours later; and fourth dose, 6.5 mg/kg, 2 hours later. These doses were used based on data from other investigations indicating that the maximum antithromboxane effect would occur at these levels.

The other group of five animals received no drugs. Coagulation parameters including platelet aggregation in 2 and 20 percent concentrations of adenosine diphosphate, prothrombin time, activated clotting time and partial thromboplastin times were measured using blood from jugular punctures. Measurements were conducted just before administration of ibuprofen and 1 hour after administration of the second dose. Control blood was drawn from the untreated sheep at the same time intervals.

All tests were performed in our coagulation laboratory at the University of California at Davis. Activated partial thromboplastin times were evaluated using rabbit brain cephalin preparation (Actin®, Dade Diagnostics) and a commercial coagulation instrument (Fibrinometer®, Becton-Dickinson). Prothrombin times were measured using a commercial reagent (Thromboplastin-C®, Dade Diagnostics) and the same instrument. Activated clotting times were performed at 37°C using diatomaceous earth. Platelet aggregation in the presence of 2 and 20 percent adenosine diphosphate was performed using an optical aggregometer (Chrono-log Corporation) and chart recorder.

Values were averaged and compared using the Student's t test and p values of significance were derived.

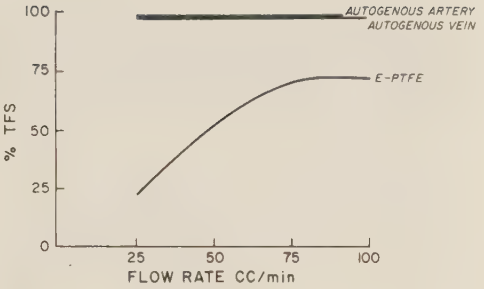

Figure 1. Four hour flow study illustrating the decrease of thrombus-free surface (TFS) of 4 mm expanded polytetrafluoroethylene (E-PTFE) at lower arterial flow rates in the sheep carotid interposition model. This was not seen with autogenous material employed in the experiment at flow rates of 25, 50, 75 and 100 cc/min.

Results

Thrombus-free surface for autogenous vein, autogenous artery and expanded polytetrafluoroethylene (Gore-Tex) was plotted at each flow rate used. The resultant thrombus-free surface values are shown for all three grafts in Table I and Figure 1. The thrombotic threshold velocity for expanded polytetrafluoroethylene was found to be 50 cc/min at 4 hours, which is comparable to the rate reported by Sauvage et al [7] for the same material using the canine model. The thrombotic threshold velocity for autogenous artery and autogenous vein was not determined in this experiment; their luminal surfaces remained essentially thrombus-free at the flow rates employed.

Using 50 cc/min as the thrombotic threshold velocity, expanded polytetrafluoroethylene was examined at 4 and 24 hours. When ibuprofen was not administered, the thrombus-free surface at 4 hours was again 50 percent. The thrombus-free surface for expanded polytetrafluoroethylene at 24 hours without ibuprofen was zero. All of these grafts were totally occluded and had their surfaces over 95 percent covered by thrombus (Figure 2).

When intravenous ibuprofen was administered, expanded polytetrafluoroethylene showed an increase in thrombus-free surface at 4 hours to an average of 87 percent (Figure 3). This differed significantly from the average thrombus-free surface of 50 percent seen without ibuprofen at 4 hours ($p < 0.01$). The grafts observed at 24 hours when ibuprofen was administered were all patent with an average thrombus-free surface of 80 percent. The thrombus deposited in the latter group was thin and relatively even over the luminal surface of the graft (Figure 4).

No significant change in coagulation parameters (activated clotting time, prothrombin time and partial thromboplastin time) was observed in this experiment whether or not ibuprofen was given.

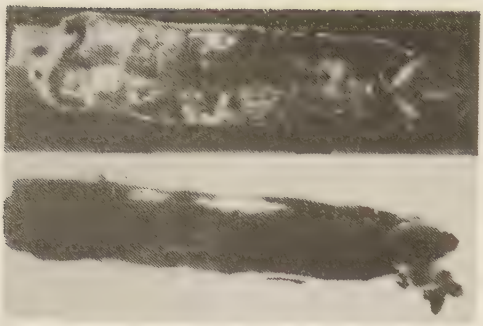

Figure 2. Luminal surface of 4 mm expanded polytetrafluoroethylene at 4 hours of in vivo flow at 50 cc/min (top) and at 24 hours of flow at 50 cc/min (bottom). The occlusive thrombosis seen at 24 hours is predictable by the extensive thrombosis seen at 4 hours.

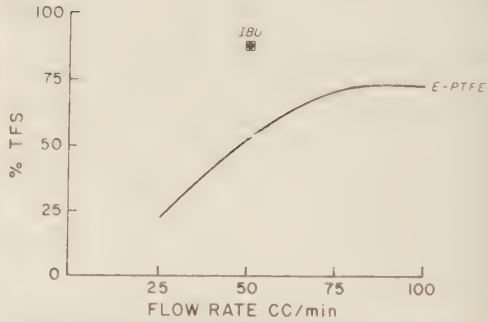

Figure 3. Four hour flow study with ibuprofen. Luminal thrombus formation decreased significantly after 4 hours of in vivo 50 cc/min flow through 4 mm expanded polytetrafluoroethylene (E-PTFE) when ibuprofen (IBU) was administered. This is compared with the graph of thrombus-free surface (TFS) versus flow rate when no drugs were administered.

There was a modest decrease in platelet aggregation in some of the animals receiving ibuprofen. With 2 percent adenosine diphosphate, aggregation under anesthesia was 65.6 ± 13.1 percent before and 57.2 ± 34.6 percent after ibuprofen. With 20 percent adenosine diphosphate, aggregation was 84.7 ± 15.3 percent before and 71.8 ± 28.2 percent after ibuprofen.

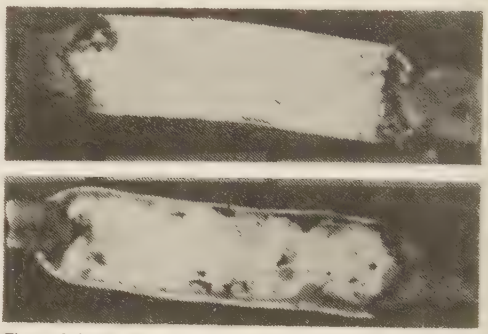

Figure 4. Luminal surface of expanded polytetrafluoroethylene (top) at 4 hours, and (bottom) at 24 hours when ibuprofen was administered. Note the lack of thrombus formation when ibuprofen was used at 50 cc/min in vivo flow compared with Figure 2, where ibuprofen was not used.

Comments

Early and intermediate-term failure of autogenous and prosthetic material in smaller (6 mm or less) diameter arterial bypass situations has largely been related to thrombosis [*3,9,11,12*]. Early thrombosis is related to thrombogenicity of prosthetic graft material, flow through the graft, mismatch of host artery-graft mechanical properties and trauma to the host artery or autogenous bypass material during grafting [*8,12–14*]. Early thrombosis appears unrelated to stenotic neointimal fibrous hyperplasia. Thromboses occurring after a few months and up to 2 years after operation appear closely related to stenotic neointimal fibrous hyperplasia near the graft origin [*3,11*]. Of these parameters the most difficult for the surgeon to control appears to be the nature of the bypass graft material.

Increasing use of available autogenous material has led to demands for synthetic materials. Glutaraldehyde-tanned umbilical veins and expanded polytetrafluoroethylene are the current synthetic materials commonly used in arterial bypasses of smaller diameter [*15,16*]. Although early reports on these materials indicated promise as a substitute for autogenous saphenous vein, long-term results have been discouraging [*17*]. A recent study reported only 24 percent patency in expanded polytetrafluoroethylene and 9 percent patency in umbilical vein grafts at 1 year even when repeated thrombectomies were performed [*13*]. Clinically and experimentally, these failures have been related to the thrombogenicity of the graft material itself, with platelet adherence and aggregation on the graft documented as a cause of this failure [*18,19*].

This experiment is our first report on the use of the in vivo thrombogenicity technique of Sauvage et al [*7*]. This has been modified for use in a sheep coagulation model that has characteristics similar to those of man and consistent coagulation parameters under conditions of experimental vascular surgery [*10*]. The results reported here illustrate that expanded polytetrafluoroethylene is a highly thrombogenic material in this model. Experimentally this appears particularly ominous in the low flow, small diameter bypass. The sheep carotid artery interposition model

has also been shown in this experiment to be useful in determining changes in thrombogenicity caused by systemic drug use. In short-term observations, this thrombogenicity is markedly reduced by ibuprofen administered intravenously.

Multiple attempts to acutely reduce graft thrombogenicity and platelet adherence and, in the longer term, stenotic neointimal fibrous hyperplasia, have been reported using systemic medication. Oblath et al [9] obtained a patency rate of 100 percent in 4 mm Dacron® and expanded polytetrafluoroethylene grafts used to bypass the femoral arteries in dogs treated with 300 mg of aspirin and 100 mg of dipyridamole daily. No thrombosis or neointimal fibrous hyperplasia was observed. In contrast, a 35 percent occlusion and a 35 percent stenosis rate in an untreated control group was observed. Acute thrombosis and stenotic neointimal fibrous hyperplasia were the causes of decreased patency rates in this group. McCann et al [20] did not obtain as good results using aspirin and dipyridamole in autogenous vein bypasses of iliac arteries in Rhesus monkeys. Occlusive neointimal fibrous hyperplasia and thrombosis was observed in 50 percent of the treated animals and in all but one of the untreated control subjects. On a weight basis, the doses used in the latter experiment were much larger than the doses used in the report of Oblath et al. Hoepp et al [21] studied the effect of corticosteroids and azathioprine on Dacron bypass grafts in the femoral arteries of dogs. Higher patency rates were observed in the azathioprine-treated group. A direct relation was found between decreased graft luminal thrombosis and the absence of neointimal fibrous hyperplasia.

Small doses of aspirin cause acetylation of the enzyme cyclooxygenase in platelets and arterial walls inhibiting thromboxane production. Thromboxane decreases cyclic adenosine monophosphate in platelets, making them more aggregable [22]. The effect of aspirin on the enzyme is dose-related, with a small dose prolonging bleeding time while a large dose does not [23]. This is probably why the results obtained by Oblath et al and McCann et al differed. Although they used the same amount of aspirin, the dogs in the experiment of Oblath et al weighed 14 to 30 kg whereas the monkeys in the experiment of McCann et al weighed 4 to 5 kg. On a dose-weight basis, the latter group falls into the large dose category and the former into the small dose category. Differences between results of these two experiments may also be explained by the combined actions of aspirin and dipyridamole [24]. Dipyridamole acts by slowing down the breakdown of cyclic adenosine monophosphate, which potentiates the inhibition of platelet aggregation by arterial wall prostacyclin. A small dose of aspirin would potentiate the beneficial effects of dipyridamole in a thrombotic situation while a large dose would inhibit prostacyclin, negating the beneficial effects of dipyridamole [24,25]. Despite the potential benefit of the use of aspirin and dipyridamole in patients with arterial bypass, clinical use has not borne out their efficacy [13].

Hoepp et al [21] felt that the beneficial effects of azathioprine and the more modest effect of steroids on increased graft patency were due to orderly intimalization of the prosthesis. Intimalization reduces the thrombogenicity of luminal prosthetic surface and subsequent occlusion. It seems equally possible that the increased patency obtained was due to decreased thromboxane production secondary to leukocyte inhibition. At present, long-term clinical use of azathioprine or steroids to increase prosthetic patency is contraindicated by the reduced healing and frequent infection noted when these drugs are used.

The beneficial effects of ibuprofen observed in this experiment appear to be a result of antithromboxane properties (data obtained from UpJohn). The intravenous form used here is experimental. As with its analgesic and anti-inflammatory effects for which it is commonly used orally in man, the precise cause of its effect on thromboxane is unknown. It has been demonstrated experimentally not to alter vessel wall prostacyclin at the doses employed in this experiment. In arthritic patients taking this drug, the main side effect seems to be gastrointestinal pain and altered motility, with ulceration and bleeding rarely occurring. Unlike potent anticoagulants such as heparin, which with prolonged use may cause bleeding, no hemorrhagic complications were observed when ibuprofen was used repeatedly. Oozing from operative sites, frequently noted with aspirin, was not observed in this experiment. Ibuprofen may be given orally, which is a desirable feature of a drug that could be used to increase the long-term patency of arterial bypass grafts. The application of ibuprofen and other antithrombotic drugs to vascular surgery in man will need to be supported by further preclinical and clinical trials.

Summary

The in vivo technique of Sauvage et al for determining prosthetic arterial graft thrombogenicity was employed in the sheep carotid model using 4 mm commercial expanded polytetrafluoroethylene (Gore-Tex) grafts. Expanded polytetrafluoroethylene was found to be thrombogenic at 4 hours of low flow in this model. At 24 hours all of these grafts were totally occluded at low flow rates. Intravenous use of ibuprofen (Motrin), a thromboxane inhibitor, in this model markedly reduced occlusion and thrombogenicity at 4 and 24 hours. All grafts were patent at 24 hours when ibuprofen was used.

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Discussion

Lester Sauvage (Seattle, WA): We have found it valuable to study experimental grafts for 6 hour periods at controlled low flow rates (50 ml/min) in the canine carotid artery. This is now our standard method of initially evaluating new candidate prostheses, since it enables us to save a good deal of time and money in weeding our grafts that are not suitable for what we are trying to accomplish. We think the method gives us substantial information about the thrombogenicity of a given flow surface, and provides that information much more rapidly than chronic implantation would. If the flow surface fares badly within the first 6 hours of exposure to blood at 50 ml/min, we do not consider it suitable for further investigation. If a prosthesis performs well in short-term, controlled flow testing, there is no guarantee it will do well as a long-term implant, but at least we can advance it to 3 week carotid implantation with some confidence. If it performs well at 3 weeks, we consider it suitable for longer-term evaluation.

Our overall program is aimed at finding reliable synthetic prostheses for the coronary arteries, and as yet we have not found such a graft. Our findings to this point can be summarized as follows: (1) Crimping of fabric grafts is detrimental if they are to be used in small caliber arteries (less than 6 mm internal diameter). (2) Precise preclotting of porous fabric prostheses is extremely important to their long-term success in small caliber environments. The four step preclotting method we have described appears useful in this regard. (3) Microporous prostheses (either expanded polytetrafluoroethylene or polyurethane) have not performed well as small caliber arterial replacements in our tests. It appears that the microporous wall undergoes what we have termed "postimplant intramural clotting" after implantation. In this process, the air in the microporous wall is replaced by blood within a few hours of implantation, followed by intramural coagulation, with release of procoagulant substances which markedly increase flow surface thrombogenicity. (4) Glutaraldehyde-preserved autograft, homograft and heterograft carotid prostheses are the least thrombogenic of all prostheses we have examined, and ones worthy of continued study as small caliber replacements. (5) Aspirin is very effective in increasing thrombus-free surface scores in all prostheses we have tested in 6 hour, low flow examinations in canines. Dr. Carson's observations of comparable effects of Motrin in the sheep are very valuable and indicate an important direction for future work. In the absence of a truly nonthrombogenic prosthetic material, we are including a wide range of antiplatelet medications in our current studies. It is becoming more apparent that any successful small caliber bypass strategy must include both a hypothrombogenic prosthesis and an effective antiplatelet regimen

Thomas Whelan (Honolulu, HI): In small diameter, low flow vessels, the thrombogenicity of the material utilized in the vascular system is more important than in larger vessels.

The experiments reported here propose to evaluate the thrombogenicity of autogenous vessels versus Gore-Tex interposed in a sheep's carotid artery and then to modify the thrombus, which was produced regularly and occluded Gore-Tex grafts at 24 hours at flow rates of 50 cc/min, with the use of Motrin, an inhibitor of platelet aggregation by inhibiting thromboxane. Under the conditions of the ex-

periment, the patency rate was increased from 0 to 100 percent with a modest decrease in platelet aggregation. This leads to my first question: since Motrin was used to decrease platelet aggregation, are the decreases in aggregation as measured significant? Perhaps it is important that this decrease in aggregation of platelets not be too marked since coagulopathy may become bothersome at other doses. It is necessary for some thrombus to form upon which neointimization may occur. Some thrombus is necessary in the healing in place of a vessel prosthesis, but it must not be excessive. How to control the amount of thrombus is the question to be answered.

The cause of late development of neointimal fibrous hyperplasia, an important cause of late closure of graft, is not known. In early work I did in dogs at Walter Reed Army Hospital, I found that although neointima might be excellent after a few months, later harvesting of the graft often showed ulcerations of the neointima, presumably due to further collaginization of the granulation tissue, choking off the fine capillary blood supply feeding the neointima. The present study, although referring to late results of small diameter prostheses of Gore-Tex, did not attempt to develop such data—only results in acute thrombosis in such prostheses.

My next question has to do with differences in species. Early in the experiments regarding vascular prostheses, great differences were apparent in dog and man. The dog appeared to intimize prostheses far better than man, and many investigators turned to the pig. Now we are told that the sheep is similar to man with respect to its coagulation parameters and platelet function. The authors apparently developed this thesis in a laboratory. Since the ultimate results of patency depend after the first week or two on other factors than thrombus formation, namely organization of the thin luminal and extragraft thrombus and intimization of the luminal thrombus, are we sure the sheep will maintain its similarity to man in these respects?

Is there an advantage of Motrin over aspirin, dipyridamole or both in effect on platelet aggregation?

This report represents an ongoing series of investigations to decrease the thrombogenicity of small diameter prosthetic grafts, in this case Gore-Tex. We need all the help we can get in order to successfully solve the small vessel replacement problem.

Jack A. Cannon (Flagstaff, AZ): It has been rightly observed that one of life's miracles is the ability of the tissue we call blood to remain in a liquid state when it is constantly exposed to a plethora of factors guaranteed to cause it to clot.

Few would deny that the luminal surface presented by healthy, confluent endothelial cells is the most antithrombogenic surface known to man. It is indeed unfortunate that human endothelium reproduces itself less efficiently than that of any other mammalian species. Thus, all patients requiring implantation of a synthetic or biosynthetic vascular prosthesis are forced to live with segments of various lengths implanted in their vascular trees with surfaces that are thrombogenic to varying degrees. So far no one, including the authors, has as yet succeeded in accurately assessing the favorable or unfavorable complications which can be predicted by measuring a thrombogenicity index.

It is an enigma to me why thrombotic flow occlusion of an abdominal aortic aneurysm is so rare. Its luminal surface is that of a multilaminated thrombus which usually thickens with the expansion of the aneurysm. Narrowing of the flow channel through this thrombus is rare.

A long-term, patent expanded polytetrafluoroethylene vascular prosthesis, described herein as possessing a highly thrombogenic surface, acquires a thin, proteinaceous luminal lining a few angstroms to a few micrometers in thickness. A preclotted, crimped Dacron vascular prosthesis on insertion presents a luminal surface which is primarily clotted blood. Its neointima gradually thickens to a few hundred micrometers and presents a nonhealed, proteinaceous surface. With larger diameters, both grafts perform well.

Some degree of thrombosis may be essential to the production of a stabilized luminal surface capable of supporting blood flow. It is interesting that endothelial cells can be efficiently grown in tissue culture provided the bottom surface of the chamber is coated with a thin film of clotted blood or plasma. Such cells can also be grown with great success if the chamber is lined with a E polytetrafluoroethylene membrane.

So far, there is no synthetic or biosynthetic vascular graft 4 mm or less in internal diameter in which a satisfactory patency-duration percentage after implantation can be maintained. This is true of the polytetrafluoroethylene vascular graft. It is interesting that 100 percent patency of long-term duration is easily obtained using 6 mm internal diameter ER polytetrafluorethylene vascular grafts. The thrombogenicity is identical in the two sizes.

Even though the authors have not convinced me of the practical significance of their sheep-artery test, I trust they will continue their endeavors. The need for better vascular grafts in the smaller diameters is tremendous.

Ronald J. Stoney (San Francisco, CA): Gore-Tex is known to have an unpredictable thrombogenicity in certain clinical applications, particularly with low flow and compromised outflow vascular beds. The authors applied the in vivo thrombogenicity test referred to by Sauvage to examine the early (4 to 24 hour) graft thrombosis tendency of this prosthesis. Nearly all early clinical arterial reconstructive failures are technical in origin rather than due to some characteristics of the implant or healing complication of the host. Nevertheless, their experimental data in sheep does suggest that the anti-inflammatory drug ibuprofen, with its known antithromboxane properties, inhibits platelet aggregation but does not alter the production of vessel wall prostacyclin. This presumably occurs by impairing the breakdown of cyclic adenosine monophosphate; thus, this drug appears to combine the benefits of low dose aspirin and dipyridamole. Whether it conveys any benefit by producing an orderly prosthetic neointimalization, thus further decreasing the thrombogenicity of the graft, is not yet known.

Before accepting this drug as the long-awaited, low risk anticoagulant that conveys extended patency to prosthetic grafts in general and to the Gore-Tex graft in high risk application in particular, several concerns should be removed.

First, this laboratory experience should be extended to analyze late graft implantation thrombotic response. Graft healing takes up to 1 year to complete, and this preliminary report fails to examine that end of the arterial healing spectrum. Further, oral rather than intravenous administration of Motrin should be studied. Second, the mechanism of this drug's apparent antiaggregation and neointimalization requires further definition and observations

in human experiences. Third, cautious, controlled preclinical and clinical trials supporting these authors' preliminary observations are in order before we should support the widespread application of this antiaggregating substance in man.

Wesley Moore (Los Angeles, CA): The authors are to be congratulated for bringing to our attention three important points: first, validation of an important thrombogenicity model; second, further demonstration of the superiority of autogenous vein over prosthetic grafts; and third, evidence that Motrin may indeed become an important part of our pharmacologic armamentarium in preventing graft thromboses. Have the authors attempted to compare polytetrafluoroethylene grafts with other available prostheses in their thrombogenicity model?

It is clear that autogenous vessels, artery and certainly vein, represent privileged conduits for doing vascular replacement. There is a good reason for this. It has been more than 100 years since a physiologist demonstrated that you could arrest circulation and not have thrombosis within normal vessels. This is due in large part to the biochemistry of endothelium; the presence of prostacyclin and antifibrinolytic substances in endothelium help normal vessels resist thrombosis. Clearly, prostheses that do not have these biochemical parameters are at a major disadvantage. On the other hand, if prosthetic grafts are the only alternative in certain circumstances, it is not fair to compare them to veins; they should be compared with other available prostheses.

Several years ago Dr. Tony Roon, working in my laboratory in San Francisco, demonstrated that polytetrafluoroethylene grafts, freshly implanted, have the lowest thrombogenicity potential of all available prostheses Perhaps the comment that polytetrafluoroethylene is a highly thrombogenic graft is too strong. What we ought to say is that compared with vein it is thrombogenic, but compared to other prostheses it is not.

Roy L. Tawes (Hillsborough, CA): Motrin seems to me to be a much safer drug than heparin and possibly safer than aspirin with dipyridamole. We have had no experience with Gore-Tex in sheep or dogs, but we have had about 50 patients since 1975 undergo Gore-Tex femorotibial and femoropopliteal bypasses to small vessels for limb salvage.

We treated all of our patients with low dose heparin, 500 units/hour for the first 48 hours, followed by aspirin and dipyridamole in some and warfarin in others. These are desperate situations for limb salvage.

I personally think Motrin is a much safer drug, and I do not know whether we really need more sheep or dog experiments. Unless some strong argument is presented, I am ready to try Motrin tomorrow.

I compliment the authors on their work. I think it is ingenious that they thought of Motrin in this respect. We have had a lot of wound hematomas with heparin in our efforts to save limbs with Gore-Tex bypass grafts. I would like to know whether they have had any wound complications; perhaps the experiment was so short that they did not.

F. William Blaisdell (closing): First, I would like to say that this is the work of Stan Carson, and I contributed very little.

Dr. Sauvage, we are indebted to you for your many years of work on graft thrombogenicity, and we recognize you as the premier investigator in this field.

We also agree that a 50 cc/min flow is probably a good point at which to test a graft, and if a graft does not remain patent with flow at that rate, it is not worth considering further as a small vessel substitute.

We have observed the thrombogenicity of grafts placed subcutaneously with some of our unusual extraanatomical bypasses. One can often predict impending failure of a graft by simply palpating it and determining its thickness. The thin, compressible grafts inevitably function well and long-term patency is the rule, whereas thicker grafts often thrombose.

Dr. Whelan, we do not believe that the antiplatelet aggregating agents will have any effect on neointimal hyperplasia in man, which is probably a result of trauma to the artery with migration of smooth muscle cells through the arterial wall of the anastomosis. It does not appear to be directly related to platelets. With regard to the species we used, we agree that the human species differs greatly from those of dogs, pigs and sheep. We have been interested in thrombotic assessment and coagulation tests, and so far it seems that of the large animals readily available, the sheep probably has a coagulation system closer to man's than any other. Whether the sheep will prove to be the best experimental animal or not, I don't know. The sheep has an advantage over the pig in that it is docile. The pig is one of the orneriest animals one can encounter, probably because it is the smartest.

Motrin is similar to other antiplatelet aggregating agents with the exception that it is not associated with a significant incidence of bleeding. With regard to antiplatelet agents, if a little is good, a lot is not necessarily better, and we are discovering, for example, some interesting facts related to the use of aspirin. A patient can be rendered asymptomatic in terms of thrombotic or embolic episodes from platelet aggregates, yet the disease proceeds unchecked when the patient is on higher doses of aspirin. This is probably because standard doses of aspirin such as 0.6 g or more several times a day block not only thromboxane, which aggregates platelets and promotes clotting time, but also vascular protective factors such as prostaglandins. Lower doses of aspirin, in theory at least, block thromboxane without blocking prostaglandin.

Dr. Cannon, we agree that the ultimate end point in artificial grafts would be endothelial resurfacing, and of course you are aware that efforts are now being made to introduce endothelium directly into vascular grafts. This can be done by scraping cells out of an associated vein and injecting these into the graft as part of the preclotting procedure. I am sure we will hear more about this in the future.

We agree that there is no satisfactory 4 mm graft. In an occasional patient we see excellent long-term results with small grafts, but certainly no uniformly successful experience has been reported.

Dr. Stoney, we certainly agree that statistics can create fake impressions but it is the best method that we as scientists have of analyzing and presenting data. Certainly, when dealing with experiments such as the type described, there can be considerable individual variation among experimental animals.

Dr. Moore, you asked whether we have compared polytetrafluoroethylene with other grafts. Dr. Carson has an active ongoing program testing various graft materials, which I hope will be the subject of future reports.

II

DECREASED ACUTE THROMBOGENICITY OF HUMAN UMBILICAL VEINS AFTER HEPARIN AND ALCOHOL TREATMENT

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Abstract

Nine glutaraldehyde-tanned human umbilical veins (GAUV) were incubated in heparin followed by storage in ethyl alcohol 50% (HAUV). These grafts were compared to 5 GAUV flushed with a heparinized solution without subsequent treatment in alcohol (HsAUV) and 4 nonheparinized GAUV (NHUV) placed in the contralateral carotid arteries of dogs for approximately three hours. The thrombogenicity was evaluated by measuring the platelet adhesion and aggregation (^{51}Cr), fibrinogen accumulation (^{125}I-fibrinogen), the thrombus-free surface (TFS) and the thrombus weight. The HAUV had the lowest ^{51}Cr radioactivity. The means of TFS and thrombus weight for the grafts were as follows: HAUV, 82% and 0.3 g (n=9); HsAUV, 53% and 0.6 g (n=5); NHUV, 13% and 1.2 g (n=4). The NHUV therefore showed the highest thrombogenicity and the HAUV the lowest. Heparinization of GAUV grafts is strongly recommended to prevent early thrombosis.

Introduction

Glutaraldehyde-tanned human umbilical veins (GAUV) have been used as arterial substitutes with some success[5] but discouraging reports about poor performance of this material have appeared in the literature[6,8,12]. The thrombogenicity of the graft material is an important factor in early failures[3]. It has recently been demonstrated[2] that fresh human umbilical veins inactivate thrombin but this function was lost when the veins were tanned with glutaraldehyde. The GAUV's regained this particular property after treatment with heparin and storage in ethyl alcohol 50%. Interestingly, the GAUV's treated with heparin, without subsequent storage in alcohol did not seem to inactivate thrombin.

The purpose of this study was to determine the acute thrombogenicity of heparin-alcohol umbilical veins (HAUV) and compare them to glutaraldehyde umbilical veins without heparin (NHUV) and glutaraldehyde umbilical veins treated with heparin but without alcohol (HsAUV).

Materials and Methods

Nine mongrel dogs of either sex with an average weight of 18 kg were studied. Autologous platelets were labelled with ^{51}chromium (^{51}Cr) and reinfused the day before the experiment[1]. Homologous ^{125}I-fibrinogen (^{125}I fib)[11] was given intravenously prior to the operation. Hematocrit, platelet count and fibrinogen were determined at the beginning and at the end of the procedure. The animals were anesthetized with sodium pentobarbital (Mebumal®), intubated and kept on a respirator at room air. Twenty centimeter lengths of umbilical vein bypass grafts were placed in the carotid arteries in a loop fashion. The anastomoses were performed end-to-side with running 6-0 polypropylene suture (Prolene®, Ethicon Inc.) and the bypassed segment of the artery was ligated adjacent to each anastomosis. A dose of 1 mg/kg i.v. of heparin (Kabi AB, Stockholm, Sweden) was given before clamping and reversed with protamine (Protamin, Kabi AB) after the last anastomosis was performed. APTT determinations before and after heparin as well as after protamine administration were obtained in order to ensure an adequate heparinization and reversal.

Using a proximal, external electromagnetic flow probe and a distal arterial occluder, the grafts were perfused at 50 ml/min in order to accelerate thrombus formation. The bypass loops were isolated with lead shields in order to avoid interference from the radioactivity emitted from the body of the animal. Determinations were made every 5 minutes for 200 seconds using an external detector (sodium iodide thallium activated scintillation counter). The radioactivity was recorded in counts per second after substracting the background radioactivity. Blood samples were obtained at 15 minute intervals throughout the experiment for determination of radioactivity. There was a marked variation in the results of platelet tag between dogs; therefore, a ratio of the counts of the grafts to counts/g of blood was calculated. After the clamps were removed, there was a lapse of 10 to 20 minutes before the first measurement of radioactivity could be done. This time was necessary for hemostasis and installing the lead shields. Grafts were removed approximately 3 hours later and the patency, thrombus free surface (TFS) and weight of the thrombus material were recorded. The TFS was calculated using a grid on high resolution photographs of the luminal surface[3].

Glutaraldehyde human umbilical veins (Meadox®-Dardik Biograft®) measuring 5 mm in diameter and 20 cm in length were used. The NHUV grafts were flushed with three liters of sterile saline solution (Natriumklorid 9 mg/ml, ACO, Sweden) just before the operation. The HsAUV's were flushed with three liters of saline solution, each liter containing 10 000 U of heparin (5000 IE/ml) (Kabi AB), followed by injection of 30 000 U of heparin (5000 IE/ml) directly into the lumen and then flushed out with saline. The HAUV's were flushed with three liters of saline, then, filled with a heparin solution (5000 IE/ml), occluded at both ends and incubated for 12 to 24 hours. The heparin was then decanted; the grafts were again flushed with a saline solution, mounted on the glass rods and stored in ethyl alcohol 50% for a variable period of time from 24 hours to 3 days. Before surgery the veins were also irrigated with 3 liters of a sterile saline solution. Using tritium-labelled heparin, a similar radioactivity/luminal surface area was found after incubation with heparin with or without subsequent alcohol treatment as well as after flushing the grafts with a heparinized solution as performed in these experiments[7].

In the first part of this study 4 HAUV's were compared to 4 NHUV's placed in the contralateral side (Group I). In the second part 5 HAUV's were compared to 5 HsAUV's also placed in the contralateral side (Group II).

The data are given as mean $\pm$ SE. The significance of difference was calculated by Student's t-test.

Results

No significant change was noticed in the Hct, platelet count and fibrinogen during the experiments. At the time of the first determination, the vein graft/blood ratio for ^{51}Cr was similar for both grafts in group I (Fig. 1), thereafter a sharp increase in the ^{51}Cr uptake, which peaked after 90 minutes and then stabilized was noticed in the NHUV's. A depression in the curve, probably related to embolization of thrombus material was observed. There was a slow increase of ^{51}Cr radioactivity in the HAUV grafts. A significant difference was found at 60, 75, 90 and 105 minutes ($p<0.05$). The graft/blood count ratio for ^{125}I-fib showed a progressive in-

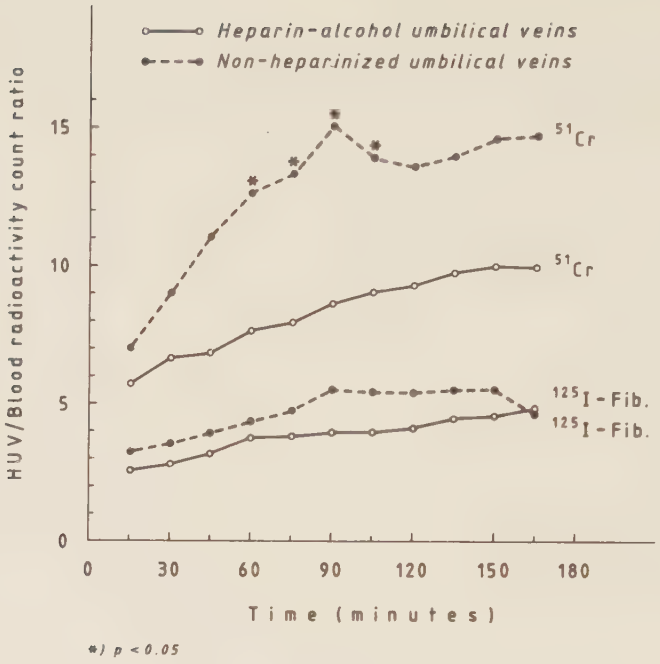

Fig. 1. Uptake of ^{51}Cr and ^{125}I-fibrinogen by heparin-alcohol and nonheparinized umbilical veins during 3 hours of observation (mean of 4 experiments).

TABLE I. Heparin-alcohol vs nonheparinized human umbilical veins (Group I). Results after 3 hours of observation. Mean and ± SE of 4 experiments.

	TFS %	Thrombus weight g	Patency %
Heparin-alcohol u.v. (n = 4)	75* (± 5 SEM)	0.5* (± 0.2 SEM)	100
Nonheparinized u.v. (n = 4)	13 (± 9 SEM)	1.2 (± 0.4 SEM)	50

TFS = Thrombus free surface; * = $p<0.005$

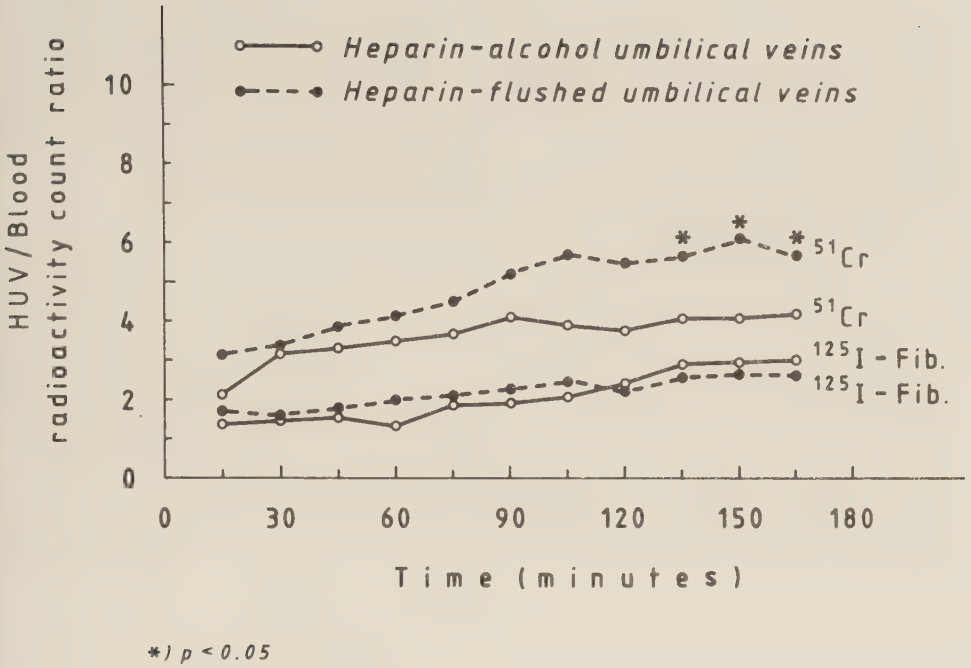

Fig. 2. Uptake of ^{51}Cr and ^{125}I-fibrinogen by heparin-alcohol and heparin without alcohol umbilical veins during 3 hours of observation (mean of 5 experiments).

crease in both types of grafts and then stabilized between 60 and 90 minutes. Eventhough there was a higher ^{125}I fib activity in the NHUV grafts, no significant difference could be demonstrated.

Two out of 4 NHUV's thrombosed 90 and 120 minutes after protamine administration whereas all HAUV's remained patent. The results regarding TFS and thrombus weight are summarized in Table I. There was a significant difference in the TFS ($p<0.05$) and thrombus weight ($p<0.05$).

The overall decreased radioactivity in group II was due to the fact that in two of the experiments, the blood flow was not restricted, but the data were included since in the same animal the two different types of grafts i.e. HAUV versus HsAUV, were compared. The HsAUV grafts had a higher count ratio for ^{51}Cr and the difference was significant at 135, 150 and 165 minutes ($p<0.05$). The ^{125}I-fib uptake was similar in both grafts (Fig. 2). None of

TABLE II. Heparin-alcohol vs heparin without alcohol human umbilical veins (Group II). Results after 3 hours of observation. Mean and ± SE of 5 experiments.

	TFS %	Thrombus weight g	Patency %
Heparin-alcohol u.v. (n = 5)	88* (± 7 SEM)	0.1 g** (± 0.09 SEM)	100
Heparin without alcohol u.v. (n = 5)	53 (± 7 SEM)	0.6 (± 0.1 SEM)	100

TFS = Thrombus free surface; * = p=0.01; ** = p=0.02

the grafts in this group thrombosed. The HAUV's had a significantly greater TFS (p=0.01) and less thrombus weight (p=0.02) than the HsAUV's (Table II). The amount of thrombus material was markedly decreased in the HAUV's and HsAUV's but in the latter it covered more luminal surface (Fig. 3). As a whole the HAUV's (9 grafts) had a TFS mean of 82% (± 5 SE), a thrombus weight mean of 0.3 g (± 0.1 SE) and 100% patency.

Discussion

Sauvage developed a model to study the thrombogenicity of graft materials for arterial substitutes and found a good correlation with their long term performance[15]. A modification of his method was used in our study to determine the adhesion and aggregation of platelets in vivo, and the accumulation of fibrinogen on the grafts tested using ^{51}Cr-labelled platelets and ^{125}I-fibrinogen, respectively. We found the NHUV's to be very thrombogenic. There was an improvement when they were flushed with a heparinized solution, but the best results were obtained when the veins were incubated with heparin followed by storage in ethyl alcohol 50%. Hufnagel[10] was able to detect 210 mg of labelled heparin/cm^2 of umbilical

Fig. 3. Luminal surface of a heparin without alcohol human umbilical vein (above) with a small amount of thrombi adhered to the wall. The luminal surface of the heparin-alcohol umbilical vein (below) is free of thrombus material.

vein surface with a concentration of heparin similar to what we used in these experiments. It seems that the binding of heparin to endothelial cells depends on the concentration of the heparin solution and to a lesser degree on the time of incubation[9,10]. Sauvage compared several prosthetic materials and during 6 hours of observation found that the TFS of umbilical veins flushed with a heparinized solution was approximately 13% at a blood flow rate of 50 ml/min[15].

Eventhough the mechanism of action is not well understood, heparin seems to exert its anticoagulant effect by facilitating the inhibitory action of antithrombin III. Furthermore, heparinized surfaces have been shown to inhibit platelet adhesion and aggregation[14]. This was also demonstrated in our experiment; nonetheless, the accumulation of fibrinogen was not altered, at least not to the same degree. Maximal platelet adhesion and aggregation occurred between 90 and 120 minutes and similar results were reported by Oblath[13].

In a previous study the fresh umbilical veins were found to adsorb thrombin which was inactivated on contact with antithrombin III. The GAUV did not inactivate thrombin, nor did HsAUV grafts. Yet this function was recovered when they were treated with ethyl alcohol 50%. It has been suggested that the beneficial effect of the alcohol could be stabilization of the heparin layer possibly by precipitation of the "structures" responsible for the heparin binding[2], this nonetheless, remains to be proved.

The present investigation demonstrates first, that heparinization of the GAUV's before implantation is necessary and second, that incubation of the GAUV's in heparin followed by storage in ethyl alcohol 50% is more effective in decreasing the acute thrombogenicity than flushing them with a heparinized solution. The HAUV grafts showed a decrease in ^{51}Cr radioactivity, a large TFS, a small amount of thrombus material and 100% patency. It is known that fresh umbilical veins are not suitable for arterial substitues due to their antigenicity and the development of aneurysms[4].

We are now investigating the effect of heparin-alcohol treatment on the long term patency and prevention of neointimal fibrous hyperplasia in GAUV grafts in addition to the assessment of the stability of the heparin-bond to the luminal surface over time.

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III

REDUCED THROMBOGENIC CHARACTERISTICS OF EXPANDED POLYTETRAFLUOROETHYLENE AND POLYURETHANE ARTERIAL GRAFTS FOLLOWING HEPARIN BONDING

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Abstract

The effect of bonding of heparin, via a glutaraldehyde stabilized ionic complex, on the early thrombogenicity of polyurethane (Hep-glut PU) and expanded polytetrafluoroethylene (Gore-Tex) (Hep-glut PTFE) as well as covalent bonding of heparin on PTFE (Cov-hep PTFE) was studied in vivo. Grafts measuring 6 cm in length and 4 mm in diameter were placed in the carotid arteries of sheep and perfused for 4 hours at 25 ml/min. The thrombogenicity was determined by calculating the percent of the luminal surface free of thrombus, thrombus weight and patency. In addition, ^{32}P-platelet accumulation was determined in some of the grafts. The stabilized ionic bonding of heparin significantly reduced the early thrombogenicity of PU but had little effect on PTFE grafts; but the thrombogenicity of the latter was markedly decreased following covalent bonding of heparin. A regional distribution of platelet accumulation was found with the distal anastomoses showing the highest platelet depósition. In conclusion, by choosing an appropriate method of heparinization, a significant reduction of the thrombogenicity of PU and PTFE grafts was achieved.

Introduction

Poor performance of small diameter vascular substitutes (<6 mm) is a frequent occurrence in low flow, high resistance locations[2]. The thrombogenicity of the graft material is a significant factor incriminated in early failures[6,10,26]. Although some improvement has been achieved by modifying the physical characteristics of the materials i.e. porosity, compliance, durability[17,29], it appears that an adjuvant antithrombotic therapy or binding of a pharmacologic agent to the surface is necessary in order to improve patency rates[2,4,19].

Platelets are major determinants of thrombus formation in the arterial system[22,23]; however, platelet compatible surfaces may still be thrombogenic by activation of the coagulation enzymes[13]. Larsson et al. demonstrated an increase in platelet and plasma-coagulation compatibility in heparinized polyethylene tubes[13].

The purpose of this work was to study the effect of heparin bonding on the early thrombogenicity of hydrophobic polymers used as arterial substitutes.

Methods and Materials

Animal model

Adult sheep of either sex with an average weight of 44 kg were studied. They were fasted for 18 hours before the experiment. After premedication with atropine, the animals were anesthetized with sodium pentobarbital (Mebumal®) and kept on a respirator with 20 % oxygen. The carotid arteries,which had an external diameter of 5-6 mm,were dissected out through a midline cervical incision. The transected artery was flushed with 5 ml of saline solution containing heparin (4 IU/ml). Grafts measuring 6 cm in length and 4 mm in diameter were interposed in the carotid arteries after removing 4 cm of the host vessel. The ends of the vessel and the graft were beveled at a 45° angle and the anastomoses were made end-to-end with running 7-0 polypropylene sutures (Prolene®, Ethicon). Using an electromagnetic flowmeter, the blood flow was recorded before and after graft placement. Grafts were perfused at 25 ml/min for 4 hours by placing a distal arterial occluder[25]. Upon removal, grafts were opened longitudinally and the luminal surface photographed. The thrombus free surface (TFS) was calculated using a grid; occluded grafts were considered to have a TFS of 0[4,26]. Thrombus weight was also recorded.

Platelet counts and APTT's were obtained before, immediately after graft placement and 30 min later.

Labelling of platelets and registration of radioactivity

Autologous platelets were labelled with ^{32}P and reinfused before graft placement[1,30]. The carotid artery was placed in a rectangular lead shield and the radioactivity was recorded using GM tubes (Philips type 18507). The lead shields were kept at body temperature by circulating warm water inside the device.

The radioactivity pulses were recorded for 200 seconds at 15 min intervals (Selectronix Scaler-Timer Model 47-21) over a period of at least 3 hours in addition to continuous recording on a pen recorder. Two independent counting channels were used for simultaneous recording of both carotid arteries. After the injection of tagged platelets the radioactivity was recorded for approximately 1 hour until it reached a steady state. Following graft placement, the activity was determined over the proximal and distal anastomoses and also over the midportion of the graft. Recordings

from the proximal carotid arteries were used as the reference points. After the clamps were removed, there was a lapse of approximately 15 min for hemostasis and installing the lead shields. Time 0 represented the time when the blood flow was restricted.

In addition, the radioactivity in the whole blood and blood fractions - platelets, plasma and corpuscular fractions - was determined 30 min after infusion of tagged platelets and then at hourly intervals for 4-6 hours. The radioactivity was measured in a β-ray analyzer (LKB-Wallac 81000, Liquid scintillation counter, Finland) and the results quench corrected[23].

Heparinization of materials

Heparin coating of the graft materials was performed by IRD Biomaterial AB (Bromma, Sweden).

Stabilized ionic bonding of heparin

Polyurethane (Cil-Monothane Elastomer, Grade Hardness Shore A-30) and expanded polytetrafluoroethylene (Gore Tex®) (PTFE) grafts were treated with a solution of polyethyleneimine and glutaraldehyde for 5 min at room temperature. This was followed by treatment with a heparin solution (0.1 mg/ml) at 50° C for 5 min. This treatment renders the surface negatively charged. Grafts were then treated with a solution containing colloidal particles of heparin and hexadecylamine-hydrochloride for 2 min at 50° C, followed by treatment with a heparin solution (0.1 mg/ml). The last two steps were repeated three times. The ionic heparin complex was finally stabilized with glutaraldehyde and rinsed with ultrapure water.

Covalent bonding of heparin

Grafts were treated with a solution of polyethyleneimine and glutaraldehyde as described above. Subsequently, the surfaces were treated with dextran sulfate (0.1 mg/ml) at 50° C for 5 min, leaving the surface negatively charged; this is followed by retreatment with polyethyleneimine. Heparin with an active aldehydo group in the terminal end of the molecule - obtained by partial degradation with nitrous acid - was bonded to the aminated surface. The reaction was performed in a water solution containing 0.4 mg/ml of heparin and 0.04 mg/ml of cyanoborohydride at a pH of 3.8 for 2 hours at 50° C.

A detailed description of these two procedures is reported elsewhere[12,13].

Experimental design

Polyurethane grafts

Stabilized ionic bonding of heparin was performed on 11 polyurethane grafts (Hep-glut PU) and compared to 10 nonheparinized polyurethane grafts (PU) with regard to TFS and thrombus weight. In 5 of these experiments, the platelet deposition was studied in Hep-glut PU and compared to PU grafts placed in the contralateral carotid arteries.

Polytetrafluoroethylene grafts

Stabilized ionic bonding of heparin was performed in 7 (Hep-glut PTFE) and covalent bonding of heparin on 8 polytetrafluoroethylene grafts (Cov-hep PTFE). The results were then compared to 5 untreated grafts (PTFE). The platelet accumulation along the graft was investigated in 6 Cov-hep PTFE grafts.

Statistical analysis

The results of TFS and thrombus weight were analyzed by Mann Whitney U test. The radioactivity values were rendered to a more normal distribution by square root transformation and the significance of difference was calculated by paired Student's t test.

TABLE I. Comparison between heparinized (Hep-glut PU) and untreated polyurethane (PU) grafts placed in the carotid arteries of sheep for 4 hours with a blood flow of 25 ml/min. ($M \pm SEM$).

Material	n=	TFS (%)	TW (mg)	Thrombosis (No)
Hep-glut PU	11	$57 \pm 10^{*}$	$82 \pm 24^{\dagger}$	1
PU	10	27 ± 8	189 ± 55	3

TFS = Thrombus free surface; TW = Thrombus weight

* $p = 0.0207$; † $p = 0.0455$

Results

There was no variation in the blood flow before and after graft placement and no change of the platelet counts or APTT's was observed.

Polyurethane grafts

The results of the PU materials are summarized in Table I. Heparinization increased the TFS, 57 ± 10 vs 27 ± 8 of the PU grafts and the difference was statistically significant (p=0.0207). The de-

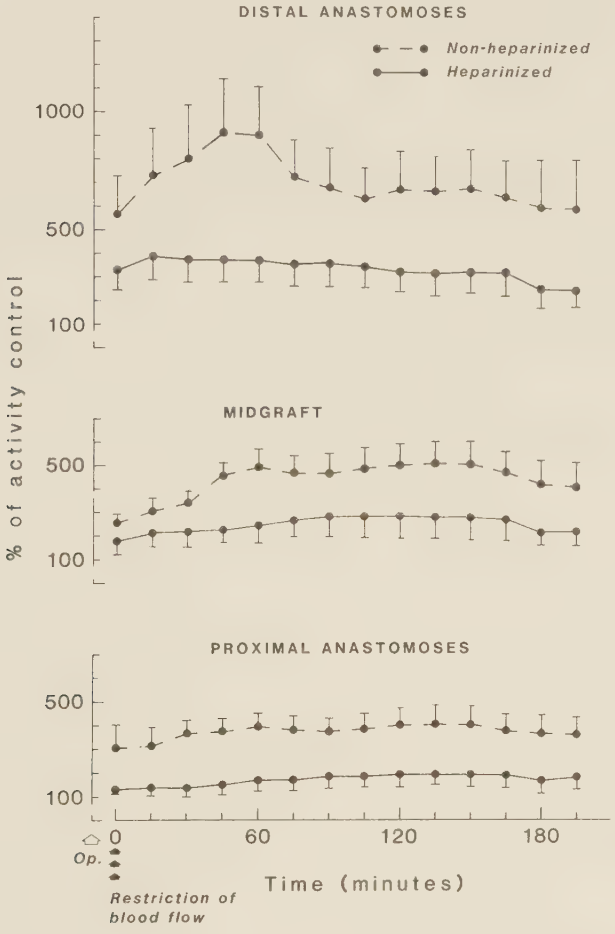

Fig. 1. Effect of stabilized ionic bonding of heparin to polyurethane grafts (Hep-glut PU) on accumulation of ^{32}P-labelled platelets compared to untreated polyurethane grafts (PU) in the proximal and distal anastomoses as well as midgraft (n=5; M ± SEM).

TABLE II. Results of covalent and stabilized ionic bonding of heparin onto PTFE grafts (Cov-hep PTFE and Hep-glut PTFE, respectively) compared to untreated PTFE grafts placed in the carotid arteries of sheep for 4 hours and perfused at 25 ml/min. ($M \pm SEM$).

Material	n=	TFS (%)	TW (mg)	Thrombosis (No)	(%)
Cov-hep PTFE	8	$57 \pm 11^{*}$	38 ± 18	1	(13)
Hep-glut PTFE	7	23 ± 12	80 ± 21	2	(30)
PTFE	5	27 ± 16	N.D.	3	(60)

N.D. = Not determined; TFS = Thrombus-free surface

* p = 0.027 vs Hep-glut PTFE; p = 0.033 vs PTFE

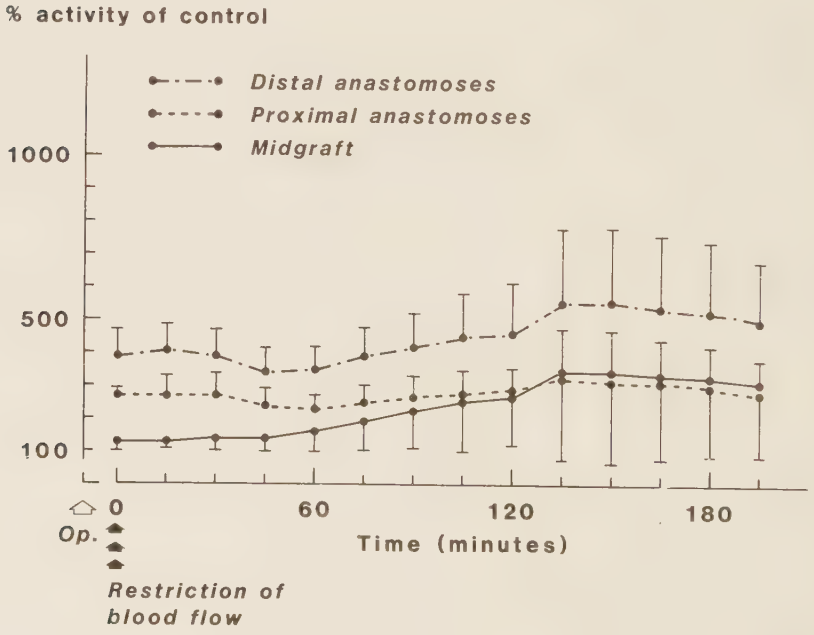

Fig. 2. ^{32}*P-platelet accumulation in the proximal and distal anastomoses and middle of covalently-heparinized PTFE (Cov-hep PTFE) grafts (n=6; M ± SEM).*

crease in the thrombus weight was of borderline significance ($p=0.0455$). Patency also improved after heparinization and only 1 out of 11 grafts thrombosed as compared to 3 out of 10 PU grafts. In this case the thrombus was not adhered to the luminal surface but to the anastomotic sites.

The radioactivity in the platelet rich plasma (PRP) fraction ranged from 75 to 88% of the whole blood activity and no significant change was noticed throughout the course of the experiments.

Compared to the PU grafts, heparinization significantly reduced platelet accumulation as shown in figure 1. Compared to the reference point values, an increase in the radioactivity from 100 to 300% occurred in the distal anastomoses of the Hep-glut PU grafts by the time the first measurement was performed. In contrast, the ^{32}P activity had increased to almost 600% in the distal anastomoses of PU grafts during the same interval and reached 900% 45 to 60 min later. Thereafter, a moderate decrease in the ^{32}P activity took place and leveled off during the rest of the period of observation. The difference was statistically significant ($p<0.05$) except in the first two determinations. The difference in the ^{32}P-platelet accumulation between the two types of grafts was of greater significance in the midgraft and proximal anastomoses; in the midgraft, the difference was not statistically significant during the first three determinations, but thenceforth the p values ranged from 0.025 to <0.005. In the proximal anastomoses the p values ranged from 0.025 to <0.005 throughout the experiment.

The deposition of tagged platelets in the distal anastomoses was higher than in the proximal anastomoses of the Hep-glut and PU grafts.

Polytetrafluoroethylene grafts

Stabilized ionic bonding of heparin improved the patency rate from 30 to 60% although the TFS remained unchanged. However, the TFS of PTFE was significantly reduced after covalent bonding of heparin and only 1 out of 8 grafts clotted (Table II).

^{32}P-platelet accumulation in the Cov-hep PTFE grafts was similar to that of Hep-glut PU grafts. A regional platelet distribution was also observed in the Cov-hep PTFE grafts and the area of the distal anastomoses showed the highest activity throughout the experiments (Fig. 2). During the first half of the experiments,

the platelet activity was higher in the proximal anastomosis than in the midgraft but in the second half the activity equalized in these two regions. During the first two hours, the ^{32}P-platelet accumulation was significantly greater in the distal anastomoses ($p<0.05$ to <0.025) and midgraft (p0.025 to 0.005).

Discussion

Expanded PTFE grafts are frequently used in patients with lower limb ischemia when autogenous saphenous veins are not available or not of good quality[3,28]. Nevertheless, this material has been shown to be thrombogenic in clinical and experimental practice[4,6]. In a recent review, Callow pointed out the fact that in low flow, high resistance locations, small diameter prostheses (<6 mm) do not perform well[2].

In part the limited success with artificial vascular prostheses is due to a lack of an adequate in vivo test which could predict graft patency. Sauvage et al.[26] developed an in vivo method to study the thrombogenicity of graft materials in dogs which was modified for use in sheep[4]. The dog does not appear to be a good animal for testing biomaterials and cardiovascular devices (NIH 80-2185 publication), mainly because of its active hemostatic and fibrinolytic systems, its very reactive vessels which lead to occlusion and the reactivity of canine platelets to heparin. Sheep have been reported to have coagulation parameters similar to man[27]. With this method modified to measure platelet deposition, we have studied the effect of heparin bonding on the thrombogenicity of PU materials and to commercially available PTFE. ^{32}Phosphorus instead of ^{51}Cr was used to label platelets since it was easier to isolate the vessel-graft activity due to the short range of the beta particles as compared to gamma radiation. The activity ranged from 75-88% in the PRP fraction and remained steady during the course of the experiments. We found that the thrombus threshold velocity, as described by Sauvage, for the PTFE grafts was 50 ml/min[4]; therefore, restriction of the flow to 25 ml/min, should cause thrombosis or heavy thrombus deposition.

Several reports have demonstrated that heparin bonding to a polymer increases platelet compatibility[11,13,21], but on the other hand, platelet compatible surfaces may still be thrombogenic

by activation of the coagulation enzymes in the cascade[13]. Larson et al. showed that hydrophobic polymer (polyethylene) furnished with a stabilized heparin ionic complex, was platelet and plasma coagulation compatible in in vitro and in vivo tests[13]. Stabilized ionic bonding of heparin reduced the early thrombogenicity of PU but had little effect on PTFE grafts. The different results are probably related to variations in the physicochemical structures of these two materials. Being less hydrophobic than the PTFE, the PU surface probably provided a better substrate for bonding of the heparin-hexadecylamine complex. On the other hand, covalent bonding of heparin did significantly decrease the thrombogenicity of PTFE grafts.

We have observed that the anastomoses create a strong stimulus for thrombus formation probably by exposure of the subendothelial structures to the circulating blood[8]. Keny et al. reported 100% patency in Goretex grafts placed in the carotid arteries of dogs 1 week after implantation, but patency dropped to 16% 2 weeks later. They suggested that factors at the anastomosis are responsible for the eventual closure, particularly in the proximal anastomoses[10].

A decreased platelet deposition was demonstrated in the heparinized PU material compared to the untreated PU. Similar findings have been reported with other heparinized polymers[11,13,21]. Reports in the literature conflict in regards to the effect of heparin on platelets[20,25]. Besides the differences in methodology, species variations and heterogenicity of heparin molecule, it appears that unbound heparin may enhance platelet activity[7,16], whereas bound heparin increases platelet compatibility[11,13,21]. Although heparinized surfaces do not prevent platelet adhesion completely, they certainly reduce platelet deposition significantly when compared to nonheparinized surfaces. It is important to emphasize that platelets are important in the healing process; therefore, and in simplistic terms, platelet adhesion should be enough to stimulate healing and not excessive to induce thrombus formation. Healing with the formation of a neointima will make the surface less thrombogenic[9].

A higher platelet activity was found in the area of the distal anastomoses compared to the midgraft and proximal anastomoses and similar results have been reported[5]. It is possible that flow turbulance at the proximal anastomosis may activate platelets by

disruption of red blood cells (RBC's) with the release of ADP, and platelets will then adhere just proximal to the distal anastomoses, another zone of turbulance. In the proximal anastomosis, the production of PGI_2, by the artery just proximal to the graft, may reduce the platelet adhesion whereas in the distal anastomosis the foreign material does not for certain have the same protective effect.

It is interesting to point out that in relation to platelet activity a quiescent laser induced injury downstream is reactivated by inflicting a fresh endothelial injury upstream following the release of ADP from injured RBC's[18]. Therefore, a similar phenomenon may occur in the presence of a vascular prosthesis.

Ionic bonding of heparin has the disadvantage of heparin loss but this has been prevented by treatment with glutaraldhyde. After an initial loss of approximately 12%, no further loss was noted for 7 days in experiments in vivo[14]. Covalently bonded heparin is more stable but appears to be less platelet compatible[15], which may be secondary to bonding of active sites resulting in decreased heparin activity. The covalent bonding used in these experiments is a new method which bonds only one end of the heparin molecule to the surface. This type of conformation allows most of the molecule to be free with the advantage of leaving more active sites available. Although it is too early to say, this method of heparinization seems promising.

The following conclusions may be drawn from the present investigation: 1) Stabilized ionic bonding of heparin reduced the early thrombogenicity of PU but not of PTFE grafts, 2) Covalent bonding of heparin reduced the thrombogenicity of PTFE grafts and 3) A regional platelet deposition occurred throughout the graft with the distal anastomotic area exhibiting the highest platelet activity.

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IV

HEPARINIZED HUMAN UMBILICAL VEIN GRAFTS: DELAYED HEPARIN DESORPTION AFTER ALCOHOL TREATMENT

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Abstract

Tanned human umbilical vein grafts (Dardik Biograft®, Meadox®) were heparinized with tritium-labelled heparin. Two methods of heparinization were undertaken: 1) incubation with heparin followed by ethyl alcohol 50% treatment and 2) incubation with heparin without alcohol. Grafts were placed in the carotid arteries of sheep and removed 10 and 90 days later. Heparin was lost in contact with blood but the alcohol treatment delayed heparin desorption significantly.

Introduction

Heparin bonding to foreign surfaces is a common method used in attempt to prevent thrombosis[2,3,6-10]. Several reports have dealt with the stability of the heparin bond to synthetic materials[2,3,6,8-10], but little information is available regarding heparin desorption from previously treated biological surfaces. Studies in vitro showed that fresh or tanned umbilical veins treated with heparin and ethyl alcohol 50% inactivated thrombin; in contrast, tanned umbilical veins without heparin or with heparin but without the alcohol did not appear to have this property[1]. The combination of heparin and alcohol significantly decreased the acute thrombogenicity of glutaraldehyde tanned umbilical vein grafts when they were tested in the animal model[4].

The purpose of this work was to assess the stability of heparin bonding to commercially available human umbilical vein grafts and its influence on patency rates and morphology.

Methods and Materials

Adult sheep of either sex and with an average weight of 55 kg were used. Except for water, they were fasted for 18 hours prior to the experiment. After premedication with atropine, the animals were anesthetized with sodium pentobarbital, (Mebumal®), intubated and kept on a respirator with 20% oxygen. The procedure was carried out under strict sterile conditions and a dose of 1.2 million units of Benzathine Penicillin G i.m. before and 12 hours after the operation was administered. The carotid arteries, and in a few ani-

mals the jugular veins were dissected out through a midline incision. Using an electromagnetic flowmeter (Carolina Medical Electronics, Inc., USA) the blood flow was measured before and after placement of the grafts and later before removal. The external diameter of the carotid arteries and jugular veins was 5-6 mm and 8-10 mm, respectively. Intravenous heparin (200 IU/kg) was given before clamping and reversal was not necessary in any of the experiments. Glutaraldehyde-tanned human umbilical veins (Dardik Biograft®, Meadox®) measuring 6 cm in length and 5-6 mm in diameter were interposed in the carotid arteries or jugular veins after removing 4 cm of the host vessel. The ends of the vessel and the graft were beveled at a 45^{o} angle and the anastomoses were made end-to-end with running 6-0 polypropylene suture (Prolene®, Ethicon). Labelled heparin (Heparin sodium salt $\left[{}^{3}H(G)\right]^{-}$, 0.5 mCi/mg, NEN Chemicals GmbH Research Products, West Germany) diluted in commercial heparin (Kabi, Stockholm, Sweden; 5000 IU/ml) with a mean molecular weight of approximately 15,000 was used for the heparinization. The half life of the isotope is 12.3 years. The final concentration obtained was 0.025 mCi/ml; however, in the course of the experiments, the amount of tagged heparin was doubled (0.05 mCi/ml) to increase the radioactivity.

Two methods of heparinization were investigated: 1) incubation with the heparin solution (5000 IU/ml) for 24 hours, then storage in ethyl alcohol 50% for a variable period from 1 to 3 days (HAUV) and 2) incubation with the same heparin solution but without the alcohol treatment (HsAUV). During heparinization the grafts were held in the air, clamped at one end, filled with the heparin solution and clamped at the other end. In this way, coating the external surface with labelled heparin was avoided. Grafts were flushed with 3 liters of isotonic saline before and 3 liters after heparinization. Before placement, the grafts were divided in 10 cm lengths and from each one, 2 to 4 rings measuring approximately 1 cm in length were removed, opened and the luminal surface measured. They were placed in 5 ml of tissue solubilizer solution (Soluene®-350) until the tissue dissolved which took 3 to 4 days. Subsequently, the liquid was alloted in 1 ml portions and diluted with 10 ml of scintillation solution (Lumagel®, Lumac, The Netherlands). The radioactivity was measured in a β-ray analyzer (LKB-Wallac 81000, Liquid scintillation counter, Finland) and the results were quench corrected[12]. Following removal of the grafts

the same procedure was carried out. In each animal both carotid arteries were used with the same type of graft, i.e. HAUV with HAUV or HsAUV with HsAUV.

This investigation was divided in two parts according to the period of observation: In the first part, the grafts were removed 10 days after surgery (Group I). Six HAUV's and 6 HsAUV's were placed in the carotid arteries and the blood flow was restricted to 50 ml/min by placing a constricting silk suture 6 cm distal to the graft. Five additional grafts (3 HAUV's and 2 HsAUV's) were placed in the jugular veins and also taken out 10 days later. The blood flow in these grafts was not determined.

In the second part (Group II), 6 HAUV's and 6 HsAUV's were kept in place for 90 days and had no restriction of the blood flow. Percutaneous transfemoral arteriograms were performed on the sixtieth postoperative day.

The following tests were obtained before and then after surgery at weekly intervals for 6 weeks and then every other week: Hct, platelet count, fibrinogen content and ex vivo platelet aggregation to ADP, collagen and thrombin. In 6 animals, blood from the jugular vein was obtained for determination of labelled heparin before surgery, then after at 5, 30, 60, 120 minutes and daily for two days.

For statistical analysis, paired Student's t test was used and the level of significance was set at $p<0.05$.

Results

No change was observed in the Hct, platelet count, fibrinogen content or platelet aggregation. There were no wound or graft infections.

Group I:

The results of residual labelled heparin are summarized in Table I. A trend towards a higher radioactivity in the HAUV's was observed but the difference was not statistically significant. The mean tritium-tagged heparin activity of the patent and thrombosed HAUV grafts was 6311 ± 892 and 6151 ± 59, respectively. The radioactivity in the patent HsAUV's was 3055 ± 595 and 6205 ± 1514 in the thrombosed grafts.

TABLE I. Patency and radioactivity of tritium-labelled heparin bound to human umbilical vein grafts after 10 days of observation. (Mean ± SEM).
Blood flow was restricted to 50 ml/min.

n=	Type	Site	Observation (days)	Thrombosis	Before cpm/cm^2	After cpm/cm^2	Residual heparin %
6	HAUV	c.a.	10	3	83858 ±21300	6231 ±401	9 ± 2
6	HsAUV	c.a.	10	4	212612 ±50128	5156 ±1176	3 ± 1
3	HAUV	j.v.	10	3	37764 ±12640	5873 ±1314	28 ± 18
2	HsAUV	j.v.	10	2	57002 ±6945	3940 ±557	7 ± 1

c.a. = carotid artery; j.v. = jugular vein.

Fig. 1. Heparin-alcohol treated human umbilical vein perfused at 50 ml/min and removed on the tenth postoperative day. Note thrombus loosely attached to the luminal surface but firmly adhered to the anastomotic sites.

The overall incidence of thrombosis of the grafts placed in the carotid arteries was 58% and no difference between the two types of heparinization could be demonstrated. Furthermore, all grafts placed in the jugular veins thrombosed despite a high heparin radioactivity and the thrombi in the occluded grafts were loosely or not adhered to the luminal surface, but firmly attached to the anastomotic areas (Fig. 1).

By gross inspection, most of the patent grafts had a clean inner surface, but in grafts with low ^{3}H-heparin activity, small mural thrombi were present.

Group II:

In this group (Table II), the HAUV's exhibited a higher ^{3}H-heparin radioactivity than HsAUV's and the difference was statistically significant ($p<0.025$). Neither in group I nor in group II, could radioactivity be detected in the peripheral blood in any of the determinations. Graft patency was similar with both types of heparin treatment. Ten of 12 grafts remained patent after 90 days of

TABLE II. Patency and radioactivity of tritium-labelled heparin bound to human umbilical vein grafts after 90 days of observation. (Mean $\pm$ SEM).
Blood flow was not restricted.

n=	Type	Site	Observation (days)	Thrombosis	Before cpm/cm^2	After cpm/cm^2	Residual heparin %
6	HAUV	c.a.	90	1	16018 $\pm$2449	5442* $\pm$775	39 $\pm$ 10*
6	HsAUV	c.a.	90	1	64328 $\pm$12031	2728 $\pm$354	6 $\pm$ 2

* $p = 0.01$ to 0.025

c.a. = carotid artery; j.v. = jugular vein.

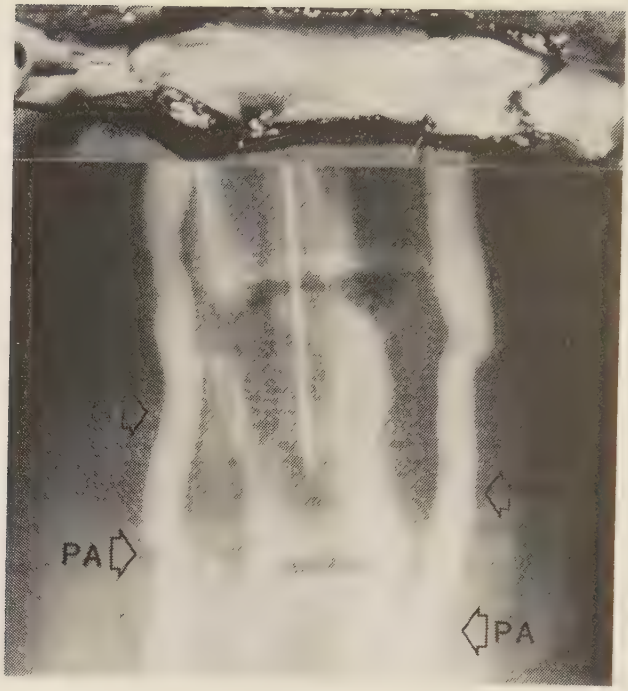

Fig. 2. Heparin-alcohol treated human umbilical vein removed 90 days after surgery. Note absence of thrombi or anastomotic lesions. Needle points to the distal anastomosis. Arteriogram on the sixtieth post-operative days shows patent grafts without anastomotic narrowing. PA = proximal anastomosis, DA = distal anastomosis and G = graft.

Fig. 3. Section from distal anastomosis of a heparin without alcohol treated umbilical vein showing thickening of the intima secondary to proliferation of smooth muscle cells, a. Disruption of the internal elastic membrane is also observed, b. Masson Trichrome, X10.

observation and the arteriograms showed no evidence of anastomotic narrowing (Fig. 2). Upon graft removal, the blood flow was 145 $\pm$ 5 ml/min among the HA-treated veins and 169 $\pm$ 23 ml/min among the HsAUV's.

Early lesions of neointimal fibrous hyperplasia were observed in the HsAUV's and less frequently in the HAUV's. These lesions appeared as shiny white areas near the anastomoses but without compromising the luminal diameter. Histologically, these lesions were characterized by proliferation of smooth muscle cells into the intima as shown in Fig. 3. In two of the HsAUV's small mural thrombi were observed in the midportion of the graft (Fig. 4).

Intimal dissection was the cause of the HAUV thrombosis.

Fig. 4. Heparin without alcohol umbilical vein graft removed 90 days after implantation showing recent mural thrombi adhered to the luminal surface and confirmed histologically. Thrombus, a; endothelial layer, b. Hematoxylin and eosin, X10. Minimal residual labelled-heparin could be detected from this graft.

Discussion

Thrombosis is a common cause of early failure of small diameter vascular prostheses and a great deal of effort has been spent in creating a thromboresistant surface. Heparin bonding to foreign surfaces has been shown to be useful in decreasing their thrombogenicity[2,3,6-10]. Numerous reports regarding heparinization of artificial prosthetic materials exist in the literature[2,3,6,8-10], but experience with binding heparin to biologic materials is limited. Glimelius, Busch and Höök demonstrated that heparin could be bound to the surface of human endothelial cells[5]. It was suggested that a limited number of binding sites is present in the endothelial cell surface since the heparin bonding was found to be time dependent and reversible and also appeared to reach a saturation point. In heparinizing umbilical vein grafts, Hufnagel observed that the amount of heparin bonded to the surface depended on the concentration of the heparin solution and little on the incubation time[7].

The present investigation was undertaken to assess the stability of the heparin bond on biomaterials and its effect on graft patency. The alcohol treatment delayed the heparin loss and the difference was statistically significant after 90 days of observation. A tendency towards a greater radioactivity in the HAUV's was already observed 10 days after graft placement. The variation in the radioactivity before placement is due to a change in the amount of labelled heparin; as explained previously, in the course of the experiments the concentration of labelled heparin was doubled in order to obtain a higher radioactivity. A great deal of heparin desorption probably occurs as soon as the heparinized surface is in contact with blood. In one animal which died 24 hours after surgery, and was not included in this series, a loss of 70% of heparin had already occurred. There seems to be a limited number of binding sites since the radioactivity in the specimens removed on the tenth postoperative day was similar in the two types of grafts. If this is the case, unbound heparin would be immediately lost as soon as the heparinized surface is in contact with blood, whereas the bond may slow down the elusion of heparin. A lower percentage of residual heparin was found in the 10-day-HAUV experiments than in the 90-day experiments although the absolute figure was higher. Since a higher concentration of labelled heparin was used in the treatment of the HAUV's in the 10-day experiments than

in the 90-day experiments, a comparison of residual heparin between the two groups is difficult. One rationale for these findings is the possibility that unlabelled heparin may have a higher affinity for the binding sites than tagged heparin. This would leave a higher concentration of the latter unbound which would be rapidly lost when in contact with the blood. Determination of tritium radioactivity in the peripheral blood yielded only background radioactivity.

No difference in the patency rate between the two types of treatment could be demonstrated. In a previous investigation, HAUV's exhibited a lower thrombogenicity compared to HsAUV's and to untreated umbilical veins; however, in those experiments there was a constant control of blood flow, and other factors such as kinking or external compression could obviously be prevented. In addition, by using labelled heparin, the HAUV's required more manipulation since they needed to be treated with alcohol and it was imperative to prevent coating of the external surface with the radioactive solution.

In the 90-day experiments, the results were very good as opposed to previous reports[11]. Despite a high heparin concentration, several grafts with restricted flow thrombosed and in these instances the thrombus was not attached to the luminal surface but firmly attached to the anastomotic sites. Similar findings have been observed in heparinized synthetic materials[8].

Early lesions of neointimal fibrous hyperplasia without compromising the luminal diameter were present particularly among the HsAUV; in contrast this lesion was the most common cause of late failure of umbilical vein grafts placed in the femoral arteries of dogs[11]. No abnormalities were observed in the platelet count, platelet aggregation or fibrinogen content, but the graft surface was probably too small to induce any significant effect.

We conclude that heparin desorption from previously treated umbilical veins is delayed by treatment with ethyl alcohol 50%; nonetheless, its mechanism of action is thus far unknown. Furthermore, thrombosis may ensue despite a high concentration of bound heparin, but in these instances the heparinized surface does not seem to be the cause of the problem, but the anastomotic site which probably creates a strong stimulus for thrombus formation.

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V

COMPARISON BETWEEN COMMERCIAL HEPARIN, LOW MOLECULAR WEIGHT HEPARIN AND PENTOSAN POLY-SULFATE ON HEMOSTASIS AND PLATELETS IN VIVO

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Abstract

The effect of commercial heparin (MW 15000; 60 U/kg), low molecular weight heparin (MW 4500, 60 U/kg), sodium pentosan polysulfate (PZ-68) in doses of 0.5, 1, 2 and 5 mg/kg and 1 mg/kg combined with dextran 70 (0.5 g/kg) on microvascular hemostasis and platelet activity in vivo was investigated in the microcirculation of rabbit mesentery and ear chamber, respectively. Low molecular weight heparin did not alter the hemostasis; nonetheless, commercial heparin and PZ-68 interfered with the hemostatic mechanism by decreasing the stability of the hemostatic plug. This abnormality was more apparent in the venular than in the arteriolar system. Both heparins exhibited a trend towards an increase in platelet reactivity and the reverse was observed following the administration of PZ-68.

Introduction

Heparin is a long chain composed of a sulfated polysaccharide polymer with variable anticoagulant activity. The reason for this lies in the fact that commercial heparin is a very heterogenous substance because of the different source of origin, chemical composition and molecular weight[1-4]. Moreover, multiple factors may influence the in vitro assays leading to conflicting results[5]. Andersson et al.[6] demonstrated that an increase in the molecular weight was associated with an increase in anticoagulant activity assessed by activated partial thromboplastin time (APTT) test. But the reverse was observed when using anti-activated factor X assay, i.e. a higher activity was found with the low molecular weight fractions. Similar results have also been reported by other investigators [7]. Contradictory results have been published of the effect of heparin on platelet aggregation in vitro[8,9]. Recently, Salzman et al.[10] reported that low molecular weight (LMW) heparin with high antithrombin III affinity did not have any significant effect on platelet activity in vitro.

Sodium pentosan polysulfate (PZ-68) is a heparin analogue which has an inhibitory effect on activated factor X and to a lesser degree on thrombin[11,12]. In a clinical investigation, Bergqvist & Ljungnér found that PZ-68 was more effective than dextran 70 in preventing deep venous thrombosis (DVT)[13]. In theory, compounds

such as LMW heparin and PZ-68 should impair thrombus formation and have a low risk of bleeding complications[6,12]. Therefore, the aim of this work was to study the effect of these two products on hemostasis and in vivo platelet activity and compare them to commercial heparin.

Materials and Methods

Microvascular hemostasis

Forty-two New Zealand rabbits of either sex with weights ranging from 2.5 to 3 kg were studied. They were anesthetized with sodium pentobarbital (Mebumal®). The microvasculature in the transparent areas of the mesentery was observed through an intravital microscope (Leitz Biomed - magnification 81x). Five arterioles and five venules measuring 20-40 μ in luminal diameter were transected before and after the administration of the drugs. The bleeding time from the proximal arteriolar segment and from either the proximal or distal venular segments was recorded as follows: primary hemostatic plug formation time (PHT), corresponding to the time between transection and the first arrest of bleeding, and the total hemostatic plug formation time (THT) which included the PHT and the sum of all rebleeding times until there was no recurrence over a 10 minute period. The frequency of rebleeding episodes was also recorded. A detailed description of the method was published elsewhere[14].

Platelet activity following a laser injury

Eleven Sandy-Lop rabbits of either sex and with an average weight of 3.5 kg were used. Titanium ear chambers were inserted 60 days prior to this investigation. Nine rabbits had suitable ear chambers with adequate regenerative tissue composed of arterioles, venules and lymphatics. After a laser injury to arterioles measuring 20-40 μ in diameter, a clump of red blood cells adhered to the endothelium at the site of injury. Platelets then began to aggregate forming a microthrombus which embolized in fragments or as a whole[15]. This phenomenon continued for several minutes and the cumulative number of emboli over a 10 minute period was recorded. Two determinations before, and thereafter at 15, 60 and 90 minutes were obtained.

Experimental design

The drugs were prepared and given intravenously by an assistant, thus the investigator was unaware which drug was being tested. The following doses were studied in both methods:

1. Commercial heparin (Batch 28336-03; M.W. = 15.000 porcine mucosa; U.S.P., 180 IU/mg, Kabi Vitrum AB, Stockholm, Sweden in doses of 60 U/kg.
2. Low molecular weight heparin (Batch No DxN10; M.W. = 4.500 porcine mucosa; U.S.P., 165 IU/mg antifactor Xa and 40 IU/mg according to APTT assay, Kabi Vitrum, in a dose of 60 U/kg anti Xa.
3. Sodium pentosan polysulfate, PZ-68; M.W. = 4.000, prepared by Benechemie, GmbH, Munich, Germany under the name of SP 54 and provided by Pharmacia AB, Uppsala, Sweden in a dose of 2 mg/kg. In the hemostasis model the following doses were also investigated: 0.5 mg/kg, 1 mg/kg, 1 mg/kg + 0.5 g/kg of dextran 70 (Macrodex, Pharmacia) and 5 mg/kg. In addition, another group of animals received 2 ml of isotonic saline (Natriumklorid ACO, 9 mg/ml, ACO Läkemedel, Sweden).

Statistical analysis

The effect on hemostasis was evaluated by comparing the results obtained after to those obtained before the administration of the drugs. The skew distribution was considered and the original values were rendered more normally distributed by logarithmic transformation. The significance of difference was evaluated by paired Student's t test. To compare between groups, the percent response of the predrug values was determined i.e. %=plug formation times after drug administration/plug formation times before drug administration x 100; the results were submitted to analysis using the Mann-Whitney U test. The difference in the frequency of rebleedings was assessed by a rank sum test which avoids assumptions on the nature of the frequency distribution of the data[14,18].

Results

Microvascular hemostasis

The results of the hemostatic plug formation time are shown in Fig. 1. Compared to venules, the arterioles exhibited a shorter

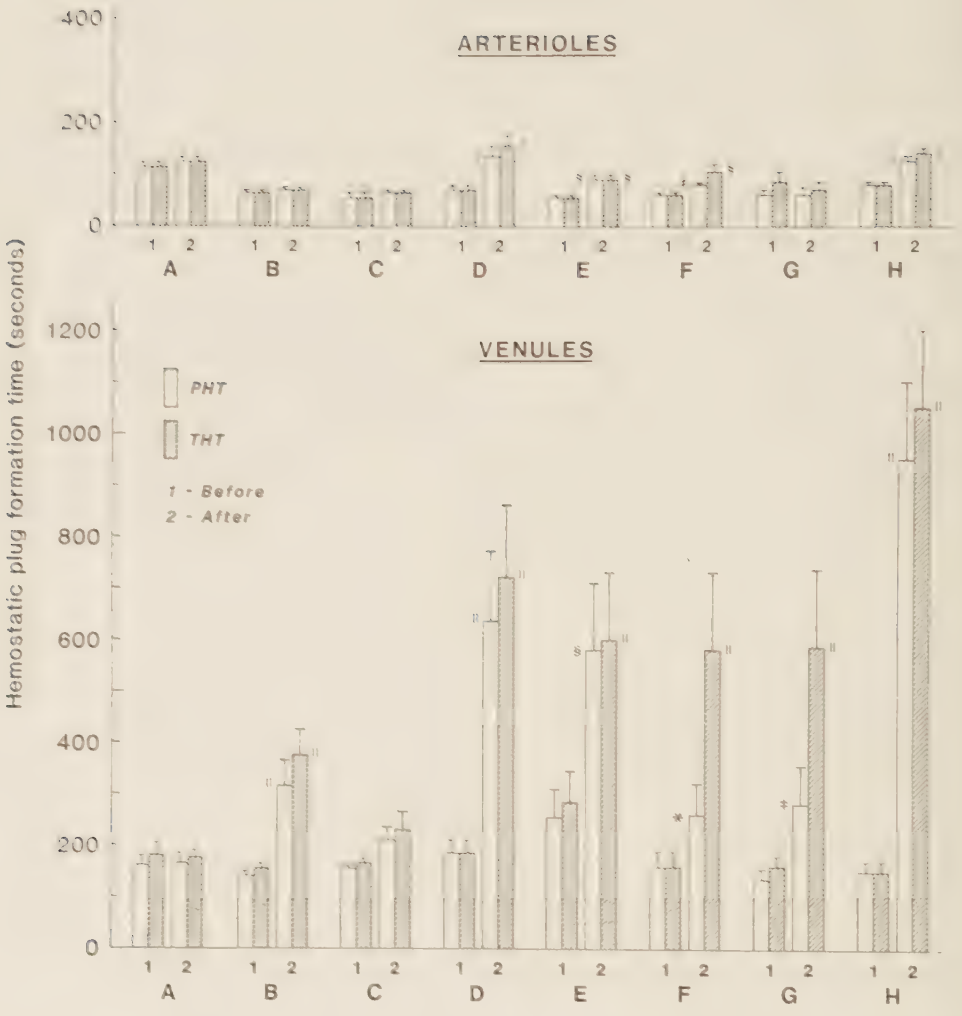

Fig. 1. Plug formation times before and after administration of: A=NaCl, n=5; B=commercial heparin, n=6; C=LMW heparin, n=6; then PZ-68 in doses of: D=0.5 mg/kg; E=1 mg/kg; F=1 mg/kg + dextran 70; G=2 mg/kg and H=5 mg/kg; n=5 in each group.
** p<0.05; ‡ p<0.025; § p<0.01 and || p<0.005. (Mean ± SE).*

PHT and THT before and after the administration of the drugs. In the arteriolar system, normal saline, both heparins and PZ-68 in a dose of 2 mg/kg did not affect the hemostatic mechanism. PZ-68 in the other doses and in combination with dextran significantly lengthened the PHT and THT. In the venules, normal saline and LMW heparin did not alter the formation and stability of the hemostatic plug and, although a tendency towards a prolonged PHT and THT was noticed after LMW heparin administration, the difference was not significant ($p=0.05$ to 0.10). PZ-68 in all doses and commercial heparin markedly prolonged the PHT and THT. A statistical evaluation comparing LMW heparin with commercial heparin and PZ-68 is shown in Table I. In the arterioles, the only significant difference which could be demonstrated occurred between LMW heparin and the smallest and highest doses of PZ-68. Moreover, no difference was observed when LMW heparin was compared to normal saline or to the intermediate doses of PZ-68 on the PHT in the venular system, but a significant difference was noted when compared to commercial

TABLE I. Statistical evaluation of low molecular weight heparin compared to commercial heparin and different doses of sodium pentosan polysulfate (PZ-68). Figures represent the p values.

	Low molecular weight heparin			
	Arterioles		Venules	
	PHT	THT	PHT	THT
Normal saline	0.2206	0.2296	0.2005	0.1587
Commercial heparin	0.1112	0.1635	0.0132	0.0023
PZ-68 0.5 mg/kg	0.0019	<0.0003	0.0188	0.0028
PZ-68 1 mg/kg	0.1814	0.1093	0.0582	0.0080
PZ-68 1 mg/kg + Dextran 70	0.0668	0.0823	0.3974	0.0427
PZ-68 2 mg/kg	0.2776	0.4325	0.3783	0.2005
PZ-68 5 mg/kg	0.0122	0.0048	<0.00003	<0.00003

TABLE II. Percentage of rebleeding episodes through previously stable hemostatic plugs.

Drugs	Arterioles		Venules	
	Before	After	Before	After
Normal saline	4	0	28	12
Commercial heparin	3	7	13	70*
LMW heparin	3	0	20	13
PZ-68 0.5 mg/kg	0	20	0	68‡
PZ-68 1 mg/kg	12	8	16	40
PZ-68 1 mg/kg + dextran	0	16	4	92§
PZ-68 2 mg/kg	4	28	28	124§
PZ-68 5 mg/kg	4	0	28	12‡

$$\text{Percentage} = \frac{\text{Number of bleeding episodes}}{\text{Number of transected vessels}} \times 100$$

* $p=0.048$, ‡ $p<0.014$, § $p<0.004$

heparin and PZ-68 except for the 2 mg/kg dose on the THT. It was also observed that PZ-68 in a dose of 2 mg/kg had a less deleterious consequence on the plug formation than smaller or larger doses but the difference was only statistically significant when compared to the dose of 0.5 mg/kg on the arterioles ($p<0.0094$) and to the 5 mg/kg dose on the arterioles and venules ($p<0.0256$ and $p<0.0007$, respectively). The frequency of rebleeding among the several groups is shown in Table II. Rebleeding episodes occurred more frequently in the venules than in the arterioles. Commercial heparin and PZ-68 in all doses augmented the frequency of rebleeding on the venular side; to the contrary, no appreciable effect in the stability of the plug was observed after the administration of LMW heparin or normal saline. The difference between LMW heparin and commercial heparin was statistically significant ($p=0.031$). Little or no effect was observed on the stability of the arteriolar hemostatic plug after the administration of the drugs.

Platelet activity

A trend towards an increased platelet reactivity was noticed following the administration of the heparins but the opposite effect was observed with PZ-68 (Fig. 2). A borderline significant difference was demonstrated between the commercial heparin and PZ-68 15 minutes after giving the drugs. Between LMW heparin and PZ-68 the difference was not statistically significant (p=0.05 to 0.10).

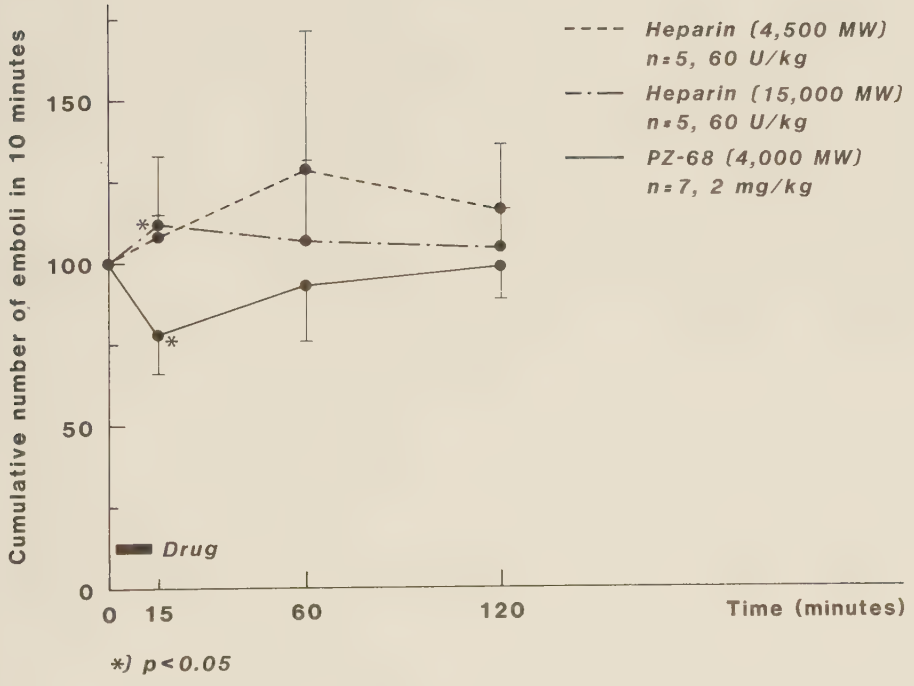

Fig. 2. Effect of heparins and pentosan polysulfate (PZ-68) on platelet activity. The cumulative number of emboli after the laser injury is expressed as the percentage of control. (Mean ± SE).

Discussion

In vitro methods are commonly used for determining the effect of drugs on platelets; nonetheless, multiple factors are known to influence the outcome. Interval time between venipuncture and performance of the test, centrifugation, type of reagents and their concentration have been implicated as causes of conflicting results[5,16,17]. Since, for obvious reasons, in vivo studies are difficult to perform in humans, animals are frequently used and although these methods are far from perfect, they provide a similar situation to that of man. The methods used in these experiments have been shown to be reliable and reproducible[14,18,19]. The primary hemostatic plug formation time (PHT) is mainly platelet dependent, whereas the total hemostatic plug formation time (THT) evaluates the stability of the plug which is the reflection of the coagulation and fibrinolytic systems[20,21].

Formation of the hemostatic plug entails a complex mechanism and it is out of the scope of this paper to discuss the possible factors involved. Nevertheless, the dissimilar plug formation times of arterioles compared to venules and the unequal response to drugs suggest that a different factor is responsible for the initiation of the hemostatic plug in the two systems. Isotonic saline and LMW heparin did not interfere with the hemostasis but commercial heparin and PZ-68 in all doses affected the hemostatic mechanism. The dose of 1 mg/kg of PZ-68 is similar to the dose of heparin chosen in these experiments in regards to their effect on F-Xa[12], and they also represent the doses used clinically in prevention of D.V.T.[13]. A dose related effect with PZ-68 on hemostasis was also investigated by using smaller and larger doses. Although in clinical practice, these compounds are administered subcutaneously, the intravenous route was chosen in our experiments to ascertain a high and similar level of circulating drug between the animals. This was desirable since little is known about the absorption of PZ-68 after the subcutaneous administration in rabbits. The main difference between LMW heparin and PZ-68 occurred in the stability of the venular hemostatic plug as shown in Table I and II. Commercial heparin and PZ-68 but not LMW heparin definitely made the platelet plug more unstable. This is not an unexpected finding since heparin with a low molecular weight fraction has a selective inhibitory action on F-Xa and little effect on thrombin; the opposite effect is observed with high mo-

lecular weight fractions[6,7]. In a previous investigation[22] heparin in doses of 150 U to 1200 U/kg i.v. lengthened the PHT and THT but the results were not statistically different from the control group. The effect indeed is similar to that reported in this investigation but the design of the study was different. In the present work, the effect was compared to the predrug values, whereas in the other study, the effect was assessed by comparing groups of animals receiving the drugs to a control group receiving no medications. PZ-68 in a dose of 75 mg (approximately 1 mg/kg) was recently reported to be more effective than dextran 70 in the prevention of postoperative DVT[13]. In that investigation the incidence of intraoperative bleeding or postoperative hematomas was not different from the dextran group. In another study[23], the administration of PZ-68 in a higher dose (100 mg twice daily) was associated with a high incidence of bleeding complications. In our experiments, the administration of PZ-68 was followed by a defective hemostatic mechanism. In a dose of 2 mg/kg, the effect was less than lower or higher doses and it is not clear why the smallest dose had a greater effect on the hemostasis than doses of 1 and 2 mg/kg and therefore needs further investigation. An increase in the fibrinolytic activity may explain the decreased plug stability observed with PZ-68 but the results are thus far conflicting[11,24].

Dextran is known to inhibit platelet function in vivo[25] but in doses not exceeding 1.5 g/kg is not associated with a prolonged bleeding time[26]. Similar results were obtained with the combination of PZ-68 (1 mg/kg) and dextran (0.5 g/kg) compared to the same dose of PZ-68 alone.

As opposed to heparins, PZ-68 showed a tendency to diminish platelet activity in the ear chamber. PZ-68 was found not to alter platelet function ex vivo[11]. Eldor and Weksler[27] reported that heparin and dextran sulfate, which is another sulfated polysaccharide, antagonize PGI_2. This finding may explain the increased platelet activity in the ear chamber induced by heparin but not the inhibitory effect of PZ-68.

The following conclusions may be drawn from this investigation: 1) Commercial heparin and PZ-68 but not low molecular weight heparin interfered with the formation and stability of the hemostatic plug, and 2) both heparins increased platelet activity whereas PZ-68 showed a tendency to decrease platelet function in vivo.

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VI

EXPERIMENTAL CAROTID STENOSIS DUE TO FIBROUS INTIMAL HYPERPLASIA

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EXPERIMENTAL CAROTID STENOSIS DUE TO FIBROUS INTIMAL HYPERPLASIA

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FOLLOWING OPERATION for atherosclerotic lesions of the extracranial carotid artery, recurrent stenosis has been observed to occur at the site of previous endarterectomy. Although these recurrences are infrequent, two distinct types of lesions have been described. Stoney and String (9), reporting a 1.5 per cent incidence of recurrent carotid stenosis, have noted that obstructing atherosclerotic plaques frequently recur more than two years after the original operation. Stenotic fibrous intimal hyperplasia occurred in their series usually less than two years after the original operation. Cossman and associates (2) reporting on carotid restenosis within 24 months of endarterectomy, found exuberant myointimal proliferation without prominent lipid deposits in all instances. Their incidence of 3.6 per cent restenosis is one of the highest ever reported. In contrast with these reports, Hertzer and associates (4) reported a 1.2 per cent recurrence of stenosis at seven months to 13 years postoperatively. Only two of these patients had myointimal proliferation without lipid deposits, one at seven months and the other at 85 months. Although fibrous intimal hyperplasia accounts for up to one-third of all reported recurrences of carotid stenosis and often leads to reoperation within two years, the cause of this lesion is unknown.

This reported experience led us to attempt to produce the lesion in a canine model. Early in the course of our experimental investigations, we found that we could not duplicate the lesion with simple carotid endarterectomy or endarterectomy with vein patch. Our subsequent investigations with morphologic and histologic findings are presented and possible causes are discussed.

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MATERIALS AND METHODS

In the first stage of this experiment, we attempted to duplicate the lesion of fibrous intimal hyperplasia by endarterectomizing the carotid ar-

FIG. 1. Lower power view of polytetrafluoroethylene graft angioplasty showing occlusion at the anastomosis. *a*, Polytetrafluoroethylene, *b*, Fibrous neointimal hyperplasia, *c*, Hematoxylin and eosin, ×2.

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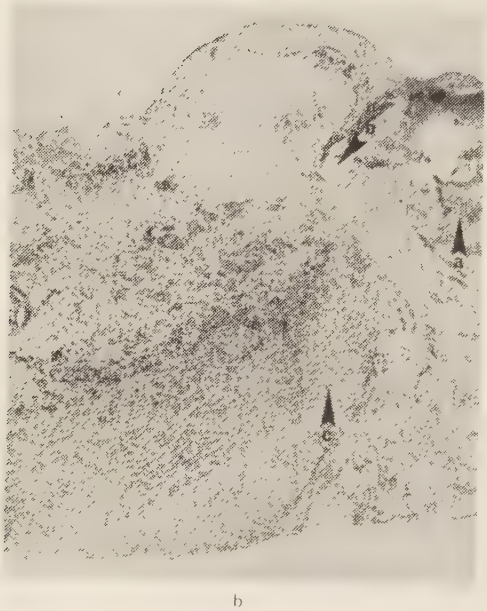
a



b

FIG. 2. a, A higher power view of artery with polytetrafluoroethylene patch angioplasty near the proximal anastomosis. Proliferation of smooth muscles, *a*, through a break in the internal elastic membrane, *b*, is noted. Trichrome stain, ×12. b, View of anastomatic area of canine carotid artery and polytetrafluoroethylene interposition graft showing fibrous intimal hyperplasia. Polytetrafluoroethylene, *a*, suture line, *b*, and fibrous neointimal hyperplasia, *c*. Hematoxylin and eosin, ×40.

teries in normal healthy mongrel dogs, weighing more than 18 kilograms. Forty carotid arteries in 20 dogs were operated upon. A simple 4 centimeter long endarterectomy with running 6-0 polypropylene suture closure were performed upon half of the carotid arteries. Identical endarterectomies were performed upon the other 20 carotid arteries but these were closed with a 4 centimeter long, 4 millimeter wide autogenous jugular vein patch using 6-0 polypropylene running sutures. All dogs received 2,000 units of heparin just prior to arterial clamping which was not reversed. Intravenously administered barbiturate was used as the anesthetic. Subsequent to completion of arterial suturing, operative angiography was performed to ensure that no significant technical errors had occurred. These 20 dogs were observed for six months and maintained on standard diet and exercise alone with no medications. At this time, all arteries were moved and examined grossly and microscopically for fibrous intimal hyperplasia. No evidence of this lesion was found in any artery and only normal endothelialization and suture line healing had occurred.

Research experience in the field of prosthetic arterial grafts led us to develop a variation of this canine carotid model in a further attempt to produce fibrous intimal hyperplasia that histologically resembled reported clinical examples. Polytetrafluoroethylene prosthetic grafts are known to cause fibrous intimal hyperplasia when used as small diameter arterial bypasses in dogs. To obtain this lesion, 4 millimeters internal diameter polytetrafluoroethylene prosthetic grafts, 4 cen-

FIG. 2. See opposite page for Figures 2a and b. c, Higher power view of section shown in Figure 2b showing proliferation of smooth muscle cells through breaks in the internal elastic membrane proximal to the anastomoses. Smooth muscle cells, *a*, and elastic membrane, *b*. Trichrome stain, ×120.

timeters in length, were placed as end-to-end interposition grafts in one carotid artery of healthy mongrel dogs weighing 18 kilograms or more. Standard vascular surgical techniques were used, including preoperative cephazolin antibiotics. Monofilament polypropylene 6-0 running sutures were used for the anastomosis. All dogs received 2,000 units of heparin prior to occlusion of the carotid arteries with umbilical tape slings. Heparin was not reversed. Intravenously given barbiturates were the only anesthetics used in the experiment.

Following a 4 centimeter endarterectomy in the contralateral carotid artery of each dog, 4 centimeters long, 4 millimeters wide longitudinal angioplasty was performed using polytetrafluoroethylene. Operative techniques were identical to those used for the end-to-end anastomosis except for the polytetrafluoroethylene patch angioplasty. This was performed without excision of any of the host artery; 6-0 monofilament polypropylene sutures were used for anastomoses.

Operative arteriograms were performed in all instances and only those dogs with anastomoses appearing technically perfect were kept in the experiment. A total of 20 dogs, 40 carotid arteries, were included in the experiment. At the end of 60 days, all dogs were sacrificed following operative angiography. Grafts and adjacent host arteries were submitted for histologic study, including transmission electron microscopy of representative samples.

RESULTS

Patency rates and causes of occlusion in the canine carotid arteries with prosthetic material were listed at 60 days. Only nine of the polytetrafluoroethylene end-to-end grafts and ten of the polytetrafluoroethylene patch angioplasties were still patent. At least one-half of the patent grafts and arteries had occlusive stenosis of 50 per cent diameter or more near their inflow anastomoses. The cause of all occlusions and every stenosis was found to be fibrous intimal hyperplasia. In the arteries that were endarterectomized with a patch angioplasty of polytetrafluoroethylene, fibrous intimal hyperplasia also occurred with thrombus ultimately occluding the residual lumen (Fig. 1). The fibrous intimal hyperplasia in these specimens was found to occur predominately at sites nearest the prosthetic material at the proximal anastomoses.

Fibrous intimal hyperplasia in the experimental models was found to consist predominately of proliferation of smooth muscle cells from the media. These were observed to be migrating to the intima through a fragmented internal elastic membrane. There was a change in the polarity of the smooth muscle cells so that their long axes were perpendicular to the lumen. An endothelial

lining was also present. These histologic changes occurred near, but not originating from, the suture line nor the areas of clamping (Fig. 2a, b and c). The internal elastic membrane never proliferated down the graft surfaces with the smooth muscle cells.

Selected samples of fibrous intimal hyperplasia occurring in the canine carotid arteries with patch angioplasty were subjected to transmission electron microscopy. Areas of gross hyperplasia near the prosthetic material and overlying the prosthetic material were studied. These confirmed the impression that fibrous intimal hyperplasia nearest to and involving the prosthetic material did not contain elastic fibers but consisted largely of smooth muscle cells lined on the intimal surface by endothelial cells with tight junctions (Fig. 3a). The areas studied from the arteries where fibrous intimal hyperplasia grossly appeared to originate showed endothelium with tight junctions along with collagen and smooth muscle cells (Fig. 3b). Unlike the hyperplasia nearest to and involving the anastomoses and prosthetic material, elastic fibrils were also present.

The results of all of our histologic studies would seem to indicate that carotid fibrous intimal hyperplasia occurring clinically and experimental fibrous intimal hyperplasia occurring secondary to prosthetic, arterial graft material insertion are similar. The lesion consists of intact endothelium overlying proliferating smooth muscle cells which make up the bulk of the lesion. A distinct elastic membrane is absent. Lipid deposits in the stenotic area in this experiment were not seen.

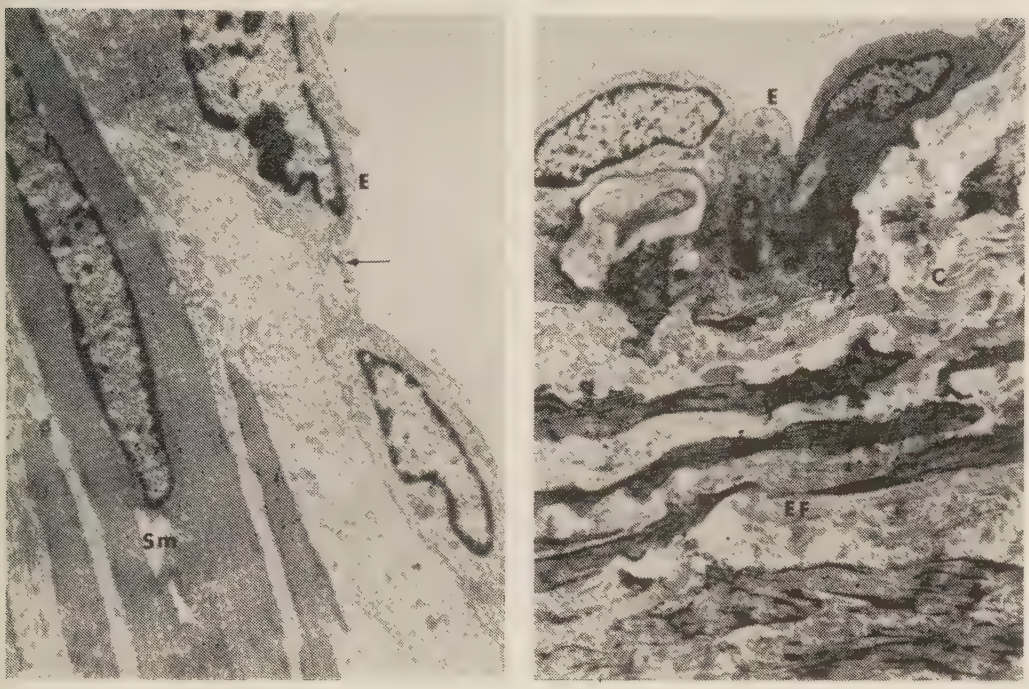
DISCUSSION

Experimental fibrous intimal hyperplasia has not previously been reported to occur in prosthetic graft arterial patch angioplasties. Our use of this model to produce the lesion was prompted by reports from Oblath (8) and Weyman (10) and their co-workers of its occurrence in experimental small diameter prosthetic grafts used in arterial impositions. The production of fibrous intimal hyperplasia by our arterial interposition prosthetic grafts is identical to that reported by Hoepp and associates (5) and others. Histologically, it appears identical to that produced by our prosthetic patch angioplasties. The histologic components are also the same as reported in clinical examples where prosthetic material was not used.

Stoney (9), French (3) and Cossman (2) and their colleagues have reported that symptomatic restenosis of the extracerebral carotid artery occurs with an incidence of 0.6 to 3.5 per cent following endarterectomy. Fibrous intimal hyperplasia and atherosclerotic plaques have been identified as a cause of restenosis. The former is usually responsible for early restenosis while the latter is said to be more frequently responsible for late stenosis. Fully one-third of these stenoses are early and are reported to be due to the lesion of fibrous intimal hyperplasia. Cossman and associates (2) reported only myointimal proliferation following carotid endarterectomy, and small diameter prosthetic graft and vein arterial bypasses in patients remain to be demonstrated. Their relationship to the lesion of intimal fibrous hyperplasia commonly observed with small diameter prosthetic arterial bypasses in experimental animals also remains to be defined. The lesion appears to occur less often in man than the canine experimental bypass. The estimated incidence in man is based on results detected by clinical evidence of recurrent ischemia following endarterectomy or arterial bypass and may be greater than that reported if symptoms are not severe. The histology of reported lesions is markedly similar and qualitatively closely resembles the smooth muscle proliferation noted after most arterial injuries. The difference between ordinary arterial injury and the lesion of intimal fibrous hyperplasia appears to be the continuous progression of smooth muscle proliferation in the latter. In addition, reformation of the internal elastic membrane does not occur in intimal fibrous hyperplasia as it does in all but the more severe arterial injuries, as demonstrated by Mansfield and colleagues (7).

The most consistent finding in our clinical and experimental data and that of others is the disruption or absence of the internal elastic membrane when fibrous intimal hyperplasia proliferates. With these histologic findings, it is difficult to believe that initial operative trauma, ischemia due to vasa vasorum disruption and immune factors or platelets are totally responsible for this lesion, particularly when an intact endothelium has been demonstrated.

Brody and co-workers (1) and others have shown that fibrous intimal hyperplasia is an important cause of failure of arterial reconstruction using autogenous veins or prosthetic materials in humans. The association between the lesions of fibrous intimal hyperplasia observed in the carotid endarterectomies and in prosthetic and autogenous graft interposition arterial replacement has not previously been described. Oblath and as-



a b

FIG. 3. a, Electron microscopic view taken from polytetrafluoroethylene patch angioplasty of endarterectomized canine carotid artery just distal to anastomosis. Absence of elastic tissue is noted. *Sm*, *Smooth muscle*. Tight junctions, arrow, between endothelial cells, *b*, are seen. ×9,470. b, View of fibrous neointimal hyperplasia taken from canine carotid artery just proximal to polytetrafluoroethylene patch angioplasty at origin of fibrous neointimal proliferation. Endothelium, *E*, with tight junctions, collagen, *C*, and elastic fibrils are identified. Dark areas in mid-portion are smooth muscle cells. *EF*, ×5,626.

sociates (8) have demonstrated that the use of aspirin and dipyridamole orally reduced the incidence of fibrous intimal hyperplasia in canine arterial prosthetic graft interposition experiments. It was believed that the aspirin and dipyridamole reduced platelet aggregation at sites of chronic endothelial damage near the prosthetic graft-host artery anastomoses. Subsequent platelet degranulation and myofibroplastic mitogensis was, therefore, prevented and fibrous intimal hyperplasia controlled. However, chronic endothelial damage was not demonstrated in the experiment. In our own experimental data, an intact endothelium without evidence of chronic damage was demonstrated. In the histologic studies performed in this experiment, smooth muscle proliferation was noted to occur at sites of breaks in the internal elastic membrane of the carotid artery. Reformation of the internal elastic membrane along the areas of the smooth muscle proliferation which comprised the bulk of fibrous intimal hyperplasia was not noted. The breaks in the internal elastic membrane were not at the actual suture lines but next to the anastomoses. Endothelium was intact over these areas.

Whether or not a common cause exists for the stenotic myointimal proliferation following carotid endarterectomy and small diameter prosthetic graft and vein arterial bypasses in patients remains to be demonstrated. Their relationship to the lesion of intimal fibrous hyperplasia commonly observed with small diameter prosthetic arterial bypasses in experimental animals also remains to be defined. The lesion appears to occur less often in man than the canine experimental bypass. The estimated incidence in man is clinical evidence of recurrent ischemia following endarterectomy or arterial bypass and may be greater than that reported if symptoms are not severe. The histology of reported lesions is markedly

similar and qualitatively closely resembles the smooth muscle proliferation noted after most arterial injuries. The difference between ordinary arterial injury and the lesion of intimal fibrous hyperplasia appears to be the continuous progression of smooth muscle proliferation in the latter. In addition, reformation of the internal elastic membrane does not occur in intimal fibrous hyperplasia as it does in all but the most severe arterial injuries as demonstrated by Mansfield and co-workers (7).

The most consistent finding in our clinical and experimental data and that of others is the disruption or absence of the internal elastic membrane when fibrous intimal hyperplasia proliferates. With these histologic findings, it is difficult to believe that initial operative trauma, ischemia due to vasa vasorum disruption and immune factors or platelets are totally responsible for this lesion, particularly when an intact endothelium has been demonstrated. Although all of these may hasten the development of fibrous intimal hyperplasia, it seems more likely that the common denominator between this lesion occurring near prosthetic arterial graft material and in autogenous endarterectomies would be a chronic hemodynamic one. Since host artery mechanical properties are not similar to the more rigid prosthetic graft materials, especially with respect to longitudinal and radical compliance, it seems possible that a mismatch in mechanical properties could also be obtained by extensively endarterectomizing a host artery. Whether this is simply a change in radial or longitudinal compliance of the endarterectomized artery with intimal and subintimal damage resulting in, or leading to, disruption of the elastic membrane with smooth muscle proliferation near the endarterectomized nonendarterectomized junction is unknown. It is likely that flow characteristics, including velocity as noted by Imparato and associates (6) may play a factor here also. This lesion has rarely been reported in carotid endarterectomies where a patch was used to close the vessel, a factor that may implicate lumen diameter and resultant flow as causats in the occurrence of intimal fibrous hyperplasia.

We agree with statements made by Imparato and co-workers (6). The formation of the intimal fibromuscular proliferation appears to be a reaction of the smooth muscle cell to unknown factors which may be hemodynamic in nature. This formation does not appear to require the chronic intervention of any blood cells or formed elements in the blood but may result from chronic damage due to mismatch in mechanical properties.

SUMMARY

Although fibrous intimal hyperplasia has been reported to be involved in up to one-third of recurrences of carotid restenosis following endarterectomy, little is known of its origin. This prompted us to attempt to produce it experimentally. Fibrous intimal hyperplasia could not be induced in the canine carotid model using simple endarterectomy or endarterectomy with an autogenous vein patch angioplasty. Polytetrafluoroethylene graft angioplasties in the same model produced this lesion readily and usually led to stenosis or occlusion. The experimental and clinical lesion of fibrous intimal hyperplasia of the carotid artery and the lesion of intimal fibrous hyperplasia that occurs when prosthetic arterial grafts are placed into small and medium size arteries appear histologically similar and may be due to a common factor. A review of the factors available in our series, as well as those of others, leads us to believe that the common denominator is hemodynamic in nature. The experimental data showing stenotic fibrous intimal hyperplasia occurring with polytetrafluoroethylene patch angioplasties has been previously reported. These data indicate that polytetrafluoroethylene should cautiously be used for small and medium diameter arterial patch angioplasties.

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VII

PROSTACYCLIN (PGI_2) PRODUCTION AND FIBRINOLYTIC ACTIVITY AFTER EXPERIMENTAL VASCULAR GRAFTING

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Abstract

The effect of arterial and venous grafting on production of prostacyclin (PGI_2) and plasminogen activator (PA) was studied. Glutaraldehyde-tanned human umbilical cord vein grafts (Dardik Biograft®, Meadox®) (HUV) measuring 6 cm in length and 5-6 mm in diameter were placed in the carotid arteries (CA's) or jugular veins (JV's) of sheep and the anastomoses were made end-to-end with running 6-0 polypropylene suture. Sections from the anastomotic sites and midgraft were submitted to PGI_2 (radioimmunoassay) and PA (histochemical) assays. Anastomotic PGI_2 synthesis from occluded HUV grafts placed in the CA's and removed on the 10th postoperative day was higher than that of control arteries; but, from occluded HUV grafts placed in the JV's, it was similar to that of control veins. From HUV grafts removed 90 days later, the anastomotic PGI_2 synthesis was similar to that of control vessels, although scanning electron microscopy frequently showed platelet and leukocyte adhesion and microthrombi at the anastomotic sites. Neointima from midgraft produced PGI_2 but less than the control vessels. All HUVG's removed 10 and 90 days later after surgery showed PA in the adventitia. In none of 11 HUVG's removed on the 10th postoperative day was there any PA in the intima; however, in 6 out of the 9 HUVG's removed on the 90th postoperative day, PA was demonstrated in the intima. In conclusion, a variable PGI_2 synthesis from the anastomotic sites was found and seemed to depend on the graft site and patency. Neointima produced PGI_2 and also gained the ability to produce PA later in time.

Introduction

The thrombogenicity of graft material and the development of anastomotic fibrous intimal hyperplasia (FIH) constitute two significant factors in the early and late failure of small diameter vascular prostheses, respectively[2,5,11,13,27].

Despite reduction of the thrombogenicity by modification of the physical characteristics of the graft material, for instance, compliance, porosity and durability[14,28] or by the administration of antiplatelet drugs or heparin bonding[3,8,18], thrombosis may still occur. Moreover, early patency can be improved by reduction of the thrombogenicity, but the grafts may eventually fail due to

the formation of FIH[2].

Since production of PGI_2 and plasminogen activator (PA) from the vascular wall are known to be important in the prevention of intravascular thrombosis[12,17], an attempt was made to study the effect of arterial and venous grafting on the two aforementioned mechanisms, particularly at the anastomotic sites.

Methods and Materials

Mature sheep of either sex with an average weight of 41 kg were used. Operations were carried out under strict sterile conditions and the animals received 1.2 million units of Benzathine Penicillin G i.m. before and 12 hours after the surgical procedure. The animals were premedicated with atropine, anesthetized with sodium pentobarbital (Mebumal®) and kept on a respirator with 20% oxygen. The procedure has been described in detail elsewhere[2,3,23] and it briefly consisted of placing interposition grafts in the carotid arteries or jugular veins after removing 4 cm of the host vessel. The ends of the vessel and the grafts were beveled at a 45^{o} angle and the anastomoses were made end-to-end with running 6-0 polypropylene suture. The external diameter of the carotid arteries and jugular veins was 5-6 mm and 8-10 mm, respectively. The blood flow was recorded with an electromagnetic flowmeter (Carolina Medical Electronics, Inc, USA) before and after graft placement and later before removal.

Glutaraldehyde-tanned human umbilical vein grafts (Dardik Biograft®, Meadox®) were used. Approximately half of the grafts were treated with a heparinized solution (5000 IU/ml, porcine mucosal heparin, Kabi, Vitrum, Stockholm) followed by treatment with ethylalcohol 50%; the rest of them were treated with the same heparin solution but without the alcohol treatment (HsAUV)[8].

Experimental design

The present investigation was divided in two parts according to the period of observation of the implanted grafts.

Intermediate-term experiments

There were two groups in these experiments. In the first group,

6 (3 HAUV and 3 HsAUV) grafts were placed in the carotid arteries and the blood flow was reduced to 50 ml/min. In the second group, 5 (3 HAUV and 2 HsAUV) grafts were placed in the jugular veins and the blood flow was not restricted. All grafts were removed on the 10th postoperative day.

Long-term experiments

Twelve (6 HAUV and 6 HsAUV) grafts were placed in the carotid arteries and removed 90 days later. Grafts were perfused at the normal physiologic blood flow.

Harvest of prostheses

Animals were anesthetized and 200 IU/kg of heparin were given i.v. before the producere. The prostheses and approximately 6 cm of the adjacent vessel proximal and distal to the grafts were removed. Patency was determined by measuring the blood flow and by direct observation of bleeding through the transected end distal to the graft. Immediately upon removal, the grafts were opened longitudinally and the luminal surface was photographed. After removing the periprosthetic tissue, sections from the anastomotic sites including a portion of the artery and a portion of the graft (half and half) as well as sections from the midgraft were tested for production of PGI_2 (Fig. 1). Midgraft specimens were analyzed for PA activity. Segments from the femoral and carotid arteries and from the jugular and femoral veins served as controls. Representative sections from the anastomotic and midgraft areas were obtained and prepared appropriately for light and scanning electron microscopy (SEM).

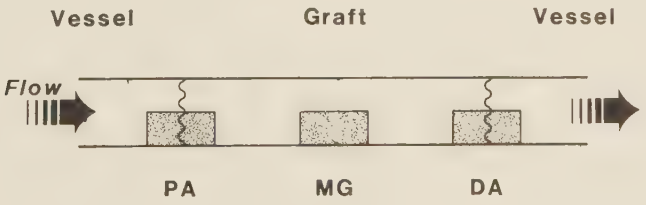

Fig. 1. Square areas represent the segments submitted for PGI_2 analysis. PA=proximal anastomoses, MG=midgraft, DA=distal anastomosis.

TABLE I. PGI_2 synthesis by segments of arteries and veins used as controls (M $\pm$ SEM).

JV n=5	FV n=4	CA n=7	FA n=7
1) 28.89 $\pm$ 7.23	1) 29.96 $\pm$ 7.93	1) 24.48 $\pm$ 2.99	1) 25.46 $\pm$ 5.96
2) 26.96 $\pm$ 3.96	2) 18.77 $\pm$ 2.78	2) 17.26 $\pm$ 4.39	2) 22.70 $\pm$ 9.14

JV=Jugular vein; FV=femoral vein; CA=carotid artery; FA= femoral artery. 1) First incubation period; 2) Second incubation period.

Prostacyclin assay

PGI_2 synthesis was assessed by measuring the amount of 6-Keto-$PGF_{1\alpha}$, a stable metabolite, in the incubation fluids by radioimmunoassay[21] (6-Keto-Prostaglandin $F_{1\alpha}$ ($^3H^+$) RIA Kit, New England Nuclear). The tissue specimens measuring 0.5 to 1 cm^2 of luminal surface were immediately rinsed with iced-cold Ringer's solution with a pH of 7.4. If present, gross thrombus material was carefully removed and this procedure took less than 2 minutes. The specimens were transferred into a fresh solution of Ringer's and incubated for 15 minutes at 37° C. Then, they were transferred again into another fresh solution and reincubated for another 15 minutes. Aliquots of the supernatant were frozen at -70° C until 6-Keto-$PGF_{1\alpha}$ assay was performed. In a few cases, samples were also tested on collagen induced platelet aggregation in vitro. The results of 6-Keto-$PGF_{1\alpha}$ are expressed in ng/ml/cm^2.

Plasminogen activator assay

For the PA assay, the specimens measuring about 1 cm in length were frozen at -70° C until subsequent analysis. The PA activity was determined using Todd's fibrin-slide technique as modified and described in detail by Pandolfi et al.[20,26]. The magnitude of PA activity was not evaluated but only whether PA activity was present or not.

TABLE II. Anastomotic and PGI_2 synthesis from heparin-alcohol treated (HAUV) and heparin without alcohol treated human umbilical veins (HsAUV) grafts. ($M \pm SEM$).

Type	HAUV	HsAUV	HAUV	HsAUV
Obs (days)	10	10	90	90
Prox anast	31.62 ± 7.16	35.05 ± 8.75	30.56 ± 9.13	25.83 ± 12.10
Mid-graft	13.60 ± 4.07	14.56 ± 7.91	11.62 ± 2.34	12.17 ± 4.10
Dist anast	63.43 ± 37.87	44.82 ± 7.70	38.34 ± 12.92	25.35 ± 8.69

Statistical analysis

The PGI_2 results were evaluated using the Mann Whitney U test and the level of significance was set at $p<0.05$.

Results

Collagen-induced aggregation was inhibited by incubating the platelets with the supernatant fluid. The results of PGI_2 production from normal arteries and veins are summarized in Table I. The PGI_2 production was slightly higher in veins than in arteries but the difference was not statistically significant. The histologic examination of these vessels showed a normal structure.

No significant difference is observed between the HAUV and the HsAUV grafts in the intermediate and long-term experiments (Table II). Furthermore, there was no difference between these two groups of grafts in regards to patency and PA; therefore, in subsequent analysis they will be considered as a whole.

Intermediate-term experiments

In the HUV grafts placed in the carotid arteries, the PGI_2 synthe-

TABLE III. PGI_2 synthesis from proximal anastomoses (PA), midgraft (MG) and distal anastomoses (DA) of HUV grafts placed in the carotid arteries of sheep for 10 days at 50 ml/min blood flow. (M ± SEM).

n=	PA	MG	DA	Patent
3	1) 24.03±1.60 2) 19.06±1.13	1) 14.54±5.83 2) 10.57±3.06	1) 32.2±11.42 2) 27.8± 7.74	Yes
3	1) 56.06±0.81 2) 39.25±4.63	1) 22.14±12.75 2) 17.07±13.47	1) 117.4±63.31 2) 58.0±50.80	No
Total 6	1) 40.05±7.21* 2) 29.15±4.99	1) 18.34±6.49 2) 13.82±6.35	1) 74.8±35.61* 2) 43.1±23.98	

1) First incubation (15 min); 2) Second incubation (15 min)

* p=0.069 vs control carotid and femoral arteries

sis from the anastomotic sites was markedly increased compared to the control arteries and almost approached statistical significance (p=0.069). A lower amount of PGI_2 was obtained from the midgraft. When the results were subgrouped according to graft patency, the anastomotic PGI_2 synthesis from patent grafts was similar to that of control arteries but from the occluded grafts, it was considerably higher but the sample size was too small for statistical evaluation (Table III).

From the HUV placed in the jugular veins, the PGI_2 production from the midgraft was significantly less than that of control veins (Table IV). The anastomotic PGI_2 synthesis was also somewhat lower than that of control veins, particularly from the distal anastomoses.

Macroscopic examination of patent grafts revealed small proliferations in the midportion of the graft and on histology, they

TABLE IV. PGI_2 synthesis from proximal anastomoses (PA), midgraft (MG) and distal anastomoses (DA) of HUV grafts placed in the jugular veins of sheep for 10 days at physiologic blood flow. (M $\pm$ SEM).

n=	PA	MG	DA	Patent
5	1) 25.25 ± 7.13	1) 8.43 ± 3.62*	1) 17.14 ± 3.75	No
	2) 18.99 ± 5.07	2) 10.81 ± 3.24†	2) 9.94 ± 2.35‡	

1) First incubation (15 min); 2) Second incubation (15 min)

* p=0.016 vs control jugular and femoral veins

† p=0.016 vs jugular veins; p=0.033 vs femoral veins

‡ p=0.008 vs jugular veins; p=0.032 vs femoral veins

were composed of fibrin with scattered inflammatory cells. An intact elastic membrane formed the lining of the graft although inflammatory cells were occasionally seen.

The occluded grafts showed thrombus material filling the conduits but the thrombi were loosely or not adhered to the luminal surface but firmly attached to the anastomoses. Microscopic examination revealed recent thrombi frequently surrounded by an acute inflammatory reaction.

Long-term experiments

Table V summarizes the results of PGI_2 from grafts removed 90 days later. The anastomotic synthesis of PGI_2 was moderately elevated compared to the control arteries. The midgraft production was less and almost reached significance (p=0.082). One graft was thrombosed and the PGI_2 content from the proximal anastomosis and midgraft was 21.2 and 8.3 ng/ml/cm^2, respectively. The PGI_2 production was not determined at the distal anastomosis because of difficulties in removing the thrombus from the vessel wall. Eleven grafts remained patent. In 3 grafts, small proliferations were

TABLE V. PGI_2 synthesis from proximal anastomoses (PA), midgraft (MG) and distal anastomoses (DA) of HUV grafts placed in the carotid arteries of sheep for 90 days at physiologic blood flow. (M ± SEM).

n=	PA	MG	DA	Patent
11	[1] 28.4±8.44 [2] 22.6±7.21	[1] 12.27±2.70* [2] 11.03±3.29	[1] 32.49±7.33 [2] 29.75±7.62	Yes
1	[1] 21.21 [2] 18.94	[1] 8.33 [2] 2.96	N.D.	No

[1] First incubation (15 min); [2] Second incubation (15 min)

N.D. = Not determined

* p=0.082 vs control carotid and femoral arteries

noted in the middle portion of the graft. Microscopic examination showed that these proliferations were composed of fibrin, chronic inflammatory cells and lined by an endothelial layer. The graft which thrombosed had an organized thrombus adhered to the luminal surface. On gross inspection of the anastomotic sites, slightly raised areas with a shiny whitish appearance were observed in the majority of the grafts. These lesions, however, did not compromize the lumen in any of the grafts. Histologically, these small lesions corresponded to early anastomotic FIH characterized by proliferation of smooth muscle cells through a disrupted internal elastic membrane. SEM also revealed that the endothelial cells overlying the anastomoses were large and pleomorphic (Fig. 2). Microscopically, most of these grafts showed an intact endothelium; however, denuded areas were also observed.

Plasminogen activator activity

All control veins and arteries showed PA activity in the adventitia.

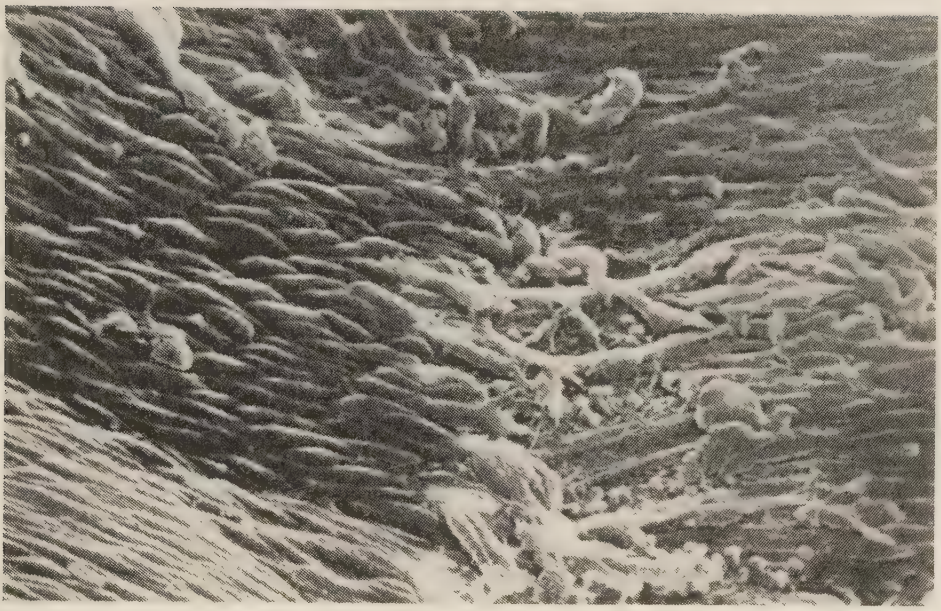

Fig. 2. Scanning electron microphotograph from the distal anastomosis of a HUV removed 90 days after surgery. The presence of pleomorphic endothelial cells and adhered platelets is observed. 300 X.

Intermediate term experiments

Eleven HUVG's placed either in the carotid arteries (n=6) or jugular veins (n=5) showed PA activity in the adventitia but in none of them was there any intimal PA activity. Three grafts placed in the carotid arteries and all grafts placed in the jugular veins thrombosed.

Long-term experiments

All grafts (n=9) showed PA's in the adventitia and in 6 grafts PA activity in the intima was demonstrated (Fig. 3). All grafts were patent.

Fig. 3. Photomicrograph of a HUV graft showing PA activity in the adventitia and in the neointima. Graft was removed 90 days later. N=neointima, A=adventitia, L=lysis.

Discussion

Failure of vascular conduits in arterial bypass surgery can arise from early or late causes. Several factors have been implicated in early failures such as poor inflow and outflow conditions, technical errors and the thrombogenicity of the graft material [1,5,13]. Late failure is frequently associated with the development of FIH [2,11]. Thrombosis is the common denominator and platelets are known to be major determinants in thrombus formation in the arterial system [19]. Platelets have also been incriminated in the etiology of FIH since administration of antiplatelet drugs prevented the development of the lesion in arterial bypass grafts in dogs [18]. We have noticed that the anastomosis creates a strong stimulus for thrombus formation since thrombus in heparinized vascular materials is not adhered to the luminal surface but firmly attached to the anastomotic sites [8]. Moreover, a high labelled platelet depo-

sition has been shown at the anastomotic areas[9,29]. Production of PGI_2 and PA from the vascular wall prevents thrombus formation by inhibition of platelet aggregation and digestion of fibrin, respectively[12,17,20]. A few reports in the literature have dealt with PGI_2 synthesis from neointima in prostheses[4,7] but there is a lack of information about the effect of vascular anastomoses on this defensive mechanism.

PGI_2 synthesis was determined by measuring the amount of 6-keto-$PGF_{1\alpha}$, a stable metabolite of prostacyclin. The specimens were incubated for 15 minutes first and subsequently reincubated for another 15 minutes trying to demonstrate any evidence of exhaustion. The PGI_2 synthesis was less in the second incubation period than in the first but the difference was not statistically significant. The incubation fluid inhibited the collagen-induced aggregation as has been reported by others[6,15]. PGI_2 production was similar in veins and arteries, and although, some investigators have found a significantly higher prostacyclin activity in arteries than in veins[24], others have reported no differences[16,25]. The discrepancy appears to be related to species variations. In our experiments, the full thickness of the vessel of graft wall was incubated in the Ringer's solution; nonetheless, most of the PGI_2 synthesis is known to come from the intima and to a lesser degree from the smooth muscle cells in the media[6,15].

A high PGI_2 synthesis from anastomotic sites of grafts placed in the carotid arteries was encountered in the occluded grafts; nonetheless, it remains unknown whether the PGI_2 production was high or low before thrombosis took place. Anastomotic areas of patent grafts from intermediate or chronic term experiments exhibited a PGI_2 synthesis similar to the control arteries. In contrast, PGI_2 production from anastomotic areas of thrombosed grafts which were placed in the jugular veins, was similar to that of control veins. Fibrin and not platelets is the main component of venous thrombi which may explain the lack of response by the PGI_2 mechanism. Grafts acquired the ability to produce PGI_2 but the amount was always less than that of normal control vessels. Clagett et al. and Eldor et al. have reported similar results from neointima in Dacron grafts and homologous vein grafts[4,7].

After 90 days, the grafts were lined by endothelial cells although occasional areas were denuded. Early lesions of anastomotic

FIH were found in several of the chronic grafts and on SEM, the endothelial cells overlying the anastomoses and vicinity were larger and pleomorphic. The cause may be hemodynamic factors leading to a persistent endothelial injury[10]. Are the "abnormal" endothelial cells capable of producing the necessary amount of PGI_2 to keep the homeostasis or do smooth muscle cells proliferate in order to supply the necessary PGI_2? These questions cannot be answered in this investigation but should be the goal of future research.

All grafts as well as normal arteries and veins exhibited fibrinolytic activity in the adventitia. As reported by others, veins showed more PA than arteries. PA occurred in areas where vasa vasorum were present. It appears that the tissue activators is transported through the vasa vasorum into the lumen of the vessel. This remains to be proved in the case of HUV grafts. None of the grafts in the intermediate term experiments showed PA in the intima, however, 6 out of 9 chronic grafts showed intimal plasminogen activator. Yates et al. and Puckett et al. in separate studies demonstrated PA in the developing neointima as early as 24 hours [22,31]. Yao et al. found no PA in occluded Dacron or bovine grafts but demonstrated PA in 3 out of 5 patent grafts[30]. They also observed that PA occurred adjacent to capillaries in the graft wall. In our experiments, we could not demonstrate intimal activity in the young grafts but with time, they gained the ability to produce PA activity.

In conclusion, arterial anastomoses produced a higher amount of PGI_2 than normal arteries in the presence of thrombus material. On the other hand, the venous anastomoses in the presence of thrombus material produced a similar or lower amount of PGI_2 than that of normal veins. Neointimal from HUV grafts acquired the ability to produce PGI_2 and PA but the latter was only observed in the long-term implanted grafts. As normal veins and arteries, HUV grafts showed PA in the adventitia but the significance of this finding needs further investigation.

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VIII

ASSESSMENT OF ANTITHROMBOTIC PROPERTIES OF SODIUM IBUPROFEN

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Abstract

The effect of sodium ibuprofen on platelet activity in vivo and the lysability of ex vivo thrombi was investigated. The formation of a hemostatic platelet plug in the rabbit mesentery and platelet embolism as a response to a laser-induced injury in the ear chamber of rabbits were used as models for determining platelet activity. Ibuprofen at a dose of 25 mg/kg i.v. was found to increase the primary (PHT) and the total hemostatic plug formation time (THT). The same dose decreased the number of cumulative emboli over a 10 min period after a laser injury to arterioles. A dose of 10 mg/kg i.v. did not affect the formation of the hemostatic platelet plug. In dogs, doses of 10, 25 and 50 mg/kg did not enhance the release of ^{125}I-FDP from the thrombi after incubation in plasmin, but the largest dose which is approximately five times the recommended dose in humans, did significantly decrease the thrombus weight 90 and 180 min after the drug administration. In conclusion, sodium ibuprofen was shown to have an inhibitory effect on platelet function in vivo and in large doses was also found to diminish the thrombus weight.

Introduction

Ibuprofen (isobutylphenylpropionic acid) like aspirin and other anti-inflammatory drugs, has been found to inhibit platelet aggregation in vitro[1-4] by altering the prostaglandin synthesis[4]. We recently demonstrated that the injectable form of ibuprofen decreased the thrombogenicity of expanded polytetrafluoroethylene grafts placed in the carotid arteries of sheep although the inhibition of platelet aggregation ex vivo was not significant[5]. These results prompted us to determine the effect of sodium ibuprofen on platelet function in vivo since multiple factors may influence the in vitro results. Moreover, it was unknown whether or not this drug had a fibrinolytic activity which could decrease the thrombogenicity of prosthetic materials without altering the platelet function and this problem was thus investigated on ex vivo thrombi[6].

Materials and Methods

Platelet function in vivo was determined from the hemostatic plug formation in rabbit mesentery and the platelet response after a biolaser-induced injury in the microcirculation of ear chambers in rabbits.

Hemostatic plug formation

Fifteen rabbits of either sex with weights ranging from 2.5 to 3 kg and anesthetized with sodium pentobarbital (Mebumal®) were studied. The method was described in detail elsewhere[7] but briefly it consisted of bringing out a short segment of the ileum through a midline incision. The mesentery was then laid over a silicone-coated glass plate mounted in a heated table and irrigated continuously with a Tyrode's solution at 38.5 $\pm$ 0.5° C. Rectal temperature was monitored and maintained between 38.5 and 39° C. An intravital microscope (Leitz Biomed) with a total magnification of 81x was employed to observe the microcirculation. Arterioles and venules of 20-40 μ in diameter were identified and transected. The time between the transection and the first arrest of hemorrhage was called the primary hemostatic plug formation time (PHT), thereafter each vessel was observed for rebleeding until there was not recurrence over a 10 min period. The sum of all bleeding times including the PHT was called the total hemostatic plug formation time (THT). Frequency of rebleeding episodes was also recorded. In each animal, five arterioles and five venules were transected before and after the drug administration. Intravenous doses of 10 mg/kg (n=5) and 25 mg/kg (n=5) of ibuprofen (sodium ibuprofen, 50 mg/ml, UpJohn, Crawley, Sussex, England) were tested. A 2 ml dose of sterile isotonic saline (Natriumklorid ACO, 9 mg/ml, ACO Läkemedel, Sweden) was administered to another five rabbits.

Platelet response after a laser injury

Eleven Sandy-Lop rabbits of either sex and with an average weight of 3.5 kg were used. Titanium ear chambers[8] were inserted 60 days prior to this investigation and the nine rabbits chosen for the experiments showed regenerative tissue with an extensive network of arterial, venous and lymphatic vessels without any occurrence of infection.

As previously described[9], a laser unit (TRG 153, New York,

USA) was assembled into a microscope (Leitz Ortholux) with a 23x intravital water immersion objective. By means of a single bio-laser pulse, arterioles with a luminal diameter of 20-40 μ were injured in unanesthetized rabbits. After the laser discharge, a burned clump of red blood cells adhered to the endothelial surface at the site of injury. Platelets then began to aggregate forming a microthrombus which embolized in fragments or as a whole. This process continued for several minutes. The cumulative number of emboli was counted over a 10 min period. Two determinations were obtained before the drug administration and after at 15, 60 and 120 min. Five drugs were tested, one of which was ibuprofen (25 mg/kg i.v.). In the two aforementioned methods, the drugs were prepared and given by an assistant, thus the investigator was unaware which drug was being tested.

Fibrinolytic activity

Fifteen adult mongrel dogs with an average weight of 18 kg were used. Homologous ^{125}I fibrinogen was given intravenously 24 hours prior to the experiment. Thrombi were formed according to Chandler[6] and the fibrinolytic activity was determined by measuring the ^{125}I fibrin degradation products (^{125}I-FDP) released by the thrombus after treatment with plasmin[10]. The thrombi were fixed in Zenker-formol solution for 4 hours, followed by rinsing in water overnight and dehydration with 80% ethylalcohol[11]. The weight of the thrombi before and after the administration of the drug was determined. The specimens were then stained with hematoxylin and eosin (H & E) and also with PTAH (phosphotungstic acid hematoxylin). For the thrombus formation, blood samples were obtained before and then at 30 and 90 min and in the animals receiving 50 mg/kg, another blood sample was drawn after 180 min. Hct, platelet count and fibrinogen tests were performed at the beginning and at the end of the experiments. Three doses of ibuprofen were tested: 10 mg/kg i.v. (n=5), 25 mg/kg i.v. (n=5) and 50 mg/kg i.v. (n=5).

Statistical analysis

The skew distribution of hemostatic plug formation times and the difference in the hemostatic pattern between the arterioles and venules were considered[7]. The frequency of rebleeding was calculated for each animal and the differences were compared using the

Wilcoxon matched-pairs signed-rank test. For the other experiments paired Student's t test was used and the level of significance was set at $p<0.05$.

Results

Effect on the hemostatic platelet plug formation

A summary of the results of ibuprofen and saline on the formation of the platelet plug is presented in Table I. Ibuprofen at a dose of 10 mg/kg i.v. did not have any effect but the larger dose (25 mg/kg) significantly increased the PHT and THT in arterioles and venules ($p<0.025$ and $p<0.01$, respectively). Rebleeding episodes occurred more frequently after ibuprofen administration particularly in the venular system ($p=0.034$) (Table II). Sterile saline administration had no effect on the hemostasis and the difference between the higher dose of ibuprofen and saline was also statistically significant ($p<0.01$).

TABLE I. Effect of ibuprofen and isotonic saline on primary (PHT) and total (THT) hemostatic plug formation times in seconds of transected arterioles and venules in the rabbit mesentery. (Mean $\pm$ SEM).

	Ibuprofen 10 mg/kg i.v. n = 5		Ibuprofen 25 mg/kg i.v. n = 5		Isotonic saline n = 5	
	PHT	THT	PHT	THT	PHT	THT
Art						
B	76 ± 10	78 ± 11	77 ± 9	77 ± 9	95 ± 10	97 ± 11
A	76 ± 11	85 ± 13	144 ± 22 [1)]	156 ± 26 [1)]	106 ± 11	106 ± 11
Ven						
B	180 ± 37	217 ± 32	118 ± 17	204 ± 85	161 ± 21	179 ± 21
A	138 ± 20	264 ± 75	356 ± 69 [2)]	502 ± 145 [2)]	161 ± 18	169 ± 17

[1)] <0.025; [2)] <0.01

Art = Arterioles; Ven = Venules; B = Before; A = After

TABLE II. Percentage of rebleeding episodes through previously stable hemostatic plugs.

	Ibuprofen 10 mg/kg		Ibuprofen 25 mg/kg		Normal saline 2 ml	
	Before	After	Before	After	Before	After
Arterioles	4	8	0	16	4	0
Venules	4	56	28	100[1])	28	12

$$\text{Percentage} = \frac{\text{Number of bleeding episodes}}{\text{Number of transected vessels}} \times 100$$

[1]) p = 0.034

Fig. 1. Effect of ibuprofen (25 mg/kg i.v.) on the cumulative number of emboli in 10 min after a laser induced injury to arterioles in the ear chamber of rabbits. Bars represent the mean and the standard error of the mean (n=6 in each group).

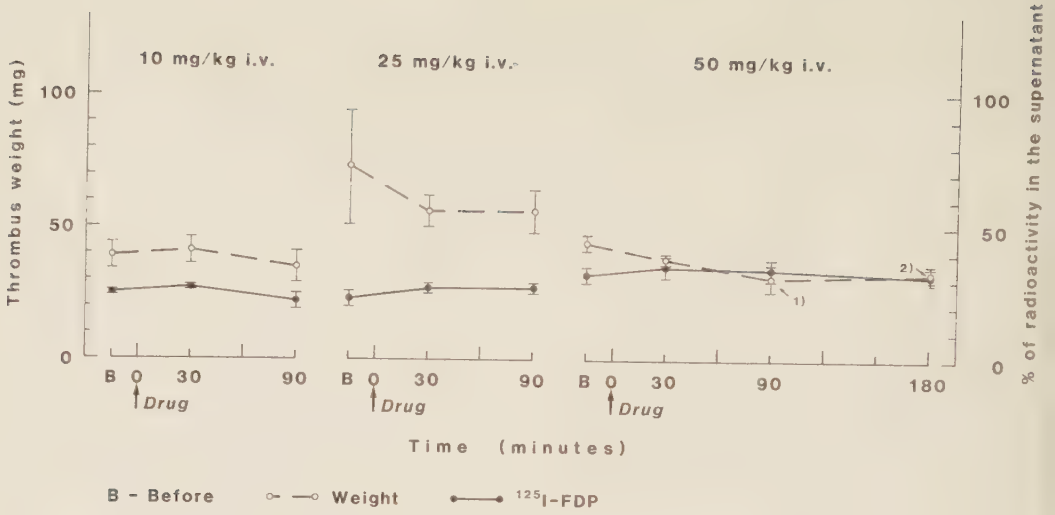

Fig. 2. Effect of several doses of ibuprofen on lysability of ex vivo thrombi. A dose of 50 mg/kg was associated with a significant decrease in the thrombus weight: [1]) p=0.01 and [2]) p<0.025 (n=5 in each group. Mean ± SEM).

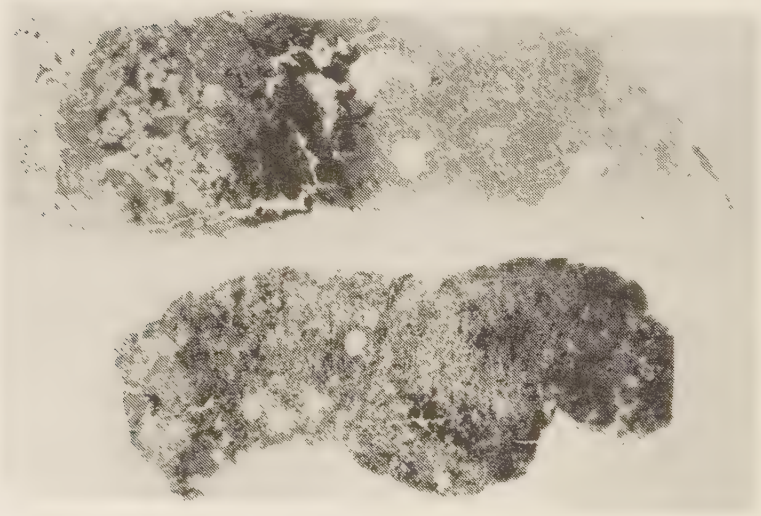

Fig. 3. Thrombi obtained before (above) and then 30 min after the administration of 50 mg/kg of ibuprofen (below). Ibuprofen changed the structure of the thrombus, formation of the head no longer occurred and the platelets became evenly distributed throughout the specimen with more erythrocytes between them. PTAH x 13.

Effect on platelet emboli after a laser injury

Fig. 1 summarizes the effect of ibuprofen 25 mg/kg i.v. on the number of emboli. A significant decrease is observed at 15 and 60 min ($p<0.025$ and $p<0.05$, respectively) after the drug administration. At 120 min, no effect on platelet activity was noticed.

Fibrinolytic activity

None of the doses tested had any effect on the release of ^{125}I-FDP from the thrombi after incubation in plasmin and no significant change in the thrombus weight was observed after the administration of 10 and 25 mg/kg of ibuprofen. However, when the dose was increased to 50 mg/kg, a significant decrease in the thrombus weight was observed 90 and 180 min after the drug administration ($p=0.01$ and $p<0.025$, respectively) (Fig. 2). Microscopic examination of the thrombi revealed some changes in their structure. In the control specimens, which were obtained before the drug administration, a typical head and a tail composed of densely packed platelets and fibrin, respectively, were observed. In contrast, after the ibuprofen administration, the head no longer formed and the platelets were distributed evenly throughout the specimen with more erythrocytes between them. These findings became more evident when the largest dose was used (Fig. 3).

No variation in the Hct, platelet count or fibrinogen content was demonstrated.

Discussion

In the arterial system, platelets constitute a very important factor in the thrombus formation as opposed to the venous system where fibrin is the main component[12]. In recent years, the use of antiplatelet drugs has increased considerably with the purpose of preventing thromboembolism from arteriosclerotic lesions and early failures in reconstructive arterial surgery, particularly in small diameter vessels[5,13-16]. In microanastomoses for instance, the aggregation of platelets can be severe enough to halt the blood flow and lead to a technical failure[17]. We recently found that sodium ibuprofen significantly impaired the thrombus formation in vascular prostheses, even though a slight inhibition of

platelet aggregation ex vivo was demonstrated[5]. Nonetheless, multiple factors may influence the in vitro results and an assessment of platelet activity in vivo was therefore necessary. Furthermore, it was important to determine whether or not ibuprofen had any thrombolytic activity which would explain the decrease in thrombogenicity of the PTFE vascular prosthesis. The formation and stability of the hemostatic platelet plug in the rabbit mesentery and the platelet response after a laser-induced injury to the microcirculation in the ear chamber of rabbits constitute two reliable and reproducible methods for the study of platelet activity in vivo[7-9]. The primary hemostatic plug formation time (PHT) is mainly platelet dependent, whereas the plug stability - determined by the total hemostatic plug formation time - reflects predominantly the status of the coagulation system[18,19].

The smaller dose of ibuprofen (10 mg/kg i.v.) did not have any effect on the formation or stability of the platelet plug but the administration of 25 mg/kg i.v. was followed by a significant increase in the PHT and THT in arterioles and venules. When the larger dose was tested in the ear chamber microcirculation, a decrease in the number of emboli was also observed. Although the mechanism responsible for the initiation of the hemostatic platelet plug is not clear, ADP and collagen seem to play a role, the latter to a lesser degree[20]. On the other hand, ADP released from the burned red blood cells seems to be responsible for the platelet thrombus formation after a laser injury[21,22].

In several ex vivo studies, ibuprofen has been found to inhibit ADP, adrenalin and collagen induced aggregation to a variable extent[1-4]. To our knowledge, however, no reports exist of its effect on platelet activity in vivo. Inhibition of the prostaglandin synthesis seems to explain its platelet inhibitory effect[4,23,24], but unlike ASA, the effect on the enzyme cyclo-oxygenase is reversible[4,24].

McIntyre, Philip and Inwood reported an increase in bleeding in normal and hemophiliac individuals on ibuprofen but it was not significant in the latter group[25]. We did not encounter any bleeding problems during our experiments in sheep; nonetheless, in the present investigation we have shown that a dose of 25 mg/kg of ibuprofen significantly prolonged the hemostatic platelet plug formation time. Caution is therefore necessary when this drug is

used in patients with bleeding tendencies.

The effect of ibuprofen (50 mg/kg) on the weight and structure of the thrombi is an interesting finding. It is probably caused by its inhibitory action on platelet function since similar findings have been reported after the administration of dextran 70 which is known to alter Factor VIII related antigen and thereby the platelet activity[26,27]. The decrease in the thrombus weight without a rise in the ^{125}I-FDP suggests that ibuprofen does not have a fibrinolytic activity per se. Nevertheless, the structural change induced by the drug probably made the thrombi more friable and the decrease in their weight was related to mechanical disruption in the plastic tubing and not to an enzymatic reaction.

In conclusion, sodium ibuprofen was shown to have an inhibitory effect on platelet function in vivo and in high doses was also found to decrease the thrombus weight. Having fewer side effects than ASA[28-30], in addition to its antithrombotic properties herewith reported, ibuprofen may be used in conditions where an antiplatelet drug regimen is necessary.

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